

Zur Kenntnis der Arylborsäuren. II*

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In der vorhergehenden Mitteilung wurden einige Umsetzungen in der *p*-Carboxy-phenylborsäure-Reihe beschrieben. Beim Nitrieren von *p*-Carboxy-phenylborsäure konnte nur die 2-Nitro-4-carboxy-phenylborsäure isoliert werden. Es wird im Folgenden die Synthese des anderen Isomeres 3-Nitro-4-carboxy-phenylborsäure III beschrieben.

Vorsichtiges Nitrieren von *p*-Tolylborsäure I bei -45 bis -40° ergab 3-Nitro-4-methyl-phenylborsäure II. Diese Säure ist schon von Bean und Johnson (1) dargestellt worden, aber wiederholte Nitrierungen nach ihren Vorschriften ergaben hauptsächlich Zersetzungsprodukte (Mono- und Dinitrotoluole). Erst bei Herabsetzen der Reaktionstemperatur wurde II in ziemlich guter Ausbeute erhalten. Die Methylgruppe liess sich mit Kaliumpermanganat in alkalischer Lösung oxydieren, wobei III entstand, mit dem eine Reihe Umsetzungen durchgeführt wurden.

Mit Methylalkohol und Chlorwasserstoff als Katalysator wurde der Carbonsäuremethylester IV und mit Phosphorpentachlorid das Säurechlorid VI gewonnen. Durch Curtiusche Reaktion wurde III zu 3-Nitro-4-amino-phenylborsäure XV abgebaut. Die B-C_{arom}-Bindung zeigte durchgehend eine beträchtliche Stabilität und Spaltprodukte konnten trotz unsanfter Behandlung nur spurenweise nachgewiesen werden. Katalytische Hydrierungen von III und V ergaben 3-Amino-4-carboxy-phenylborsäure VII bzw. 3-Amino-phenylborsäure-4-carbonsäureamid X.

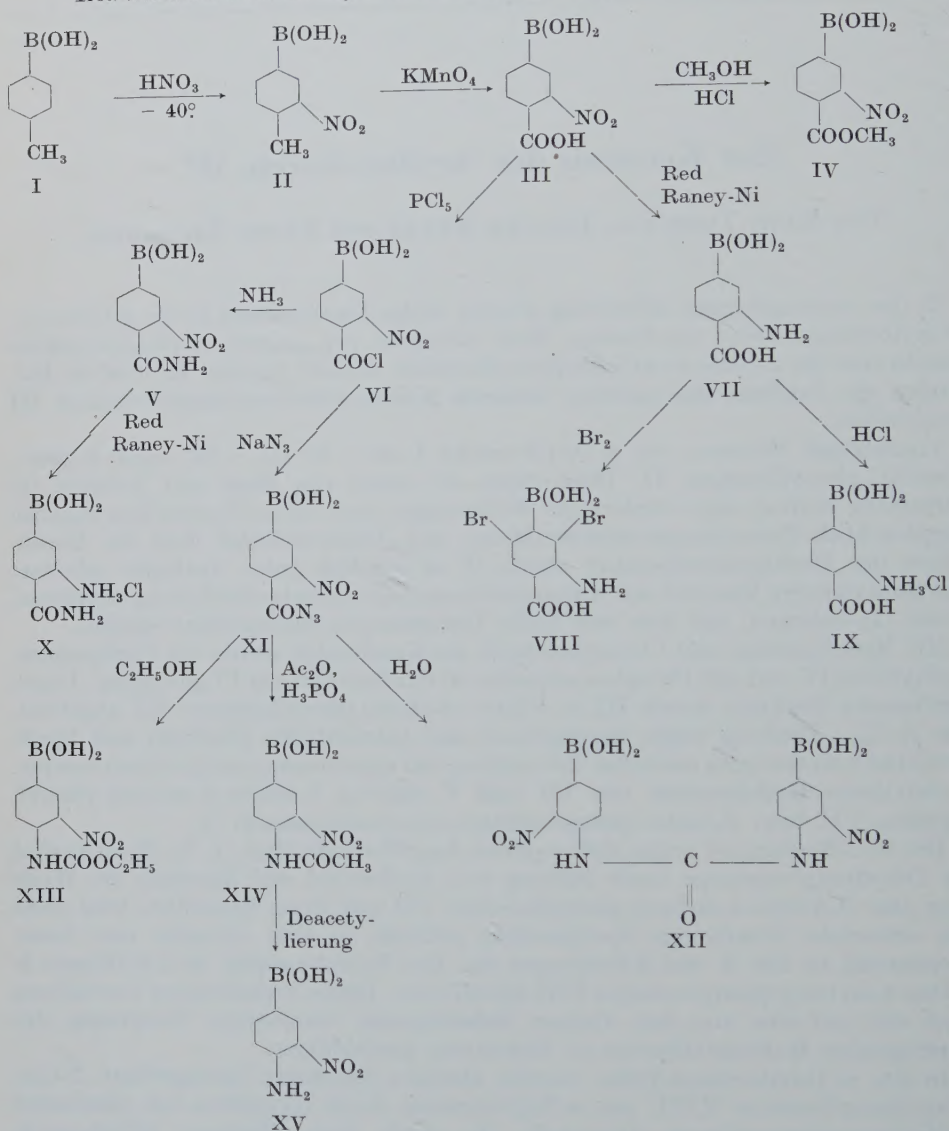
Die B-C-Bindung ist gegen elektrophilen Angriff empfindlich (1, 2). Brom spaltet die Dihydroxyborgruppe unter Bildung von Arylbromid und Borsäure ab. Wenn man aber 3-Amino-4-carboxy-phenylborsäure VII mit Brom behandelt, wird nicht das erwartete bromhaltige Spaltprodukt gebildet, es tritt vielmehr eine Kernbromierung in den 2- und 6-Stellungen ein. Das Produkt wurde als 2,6-Dibrom-3-amino-4-carboxy-phenylborsäure VIII identifiziert. Dieses Verhalten der Verbindung lässt sich auf eine von den übrigen Substituenten verursachte Verarmung des bortragenden Kohlenstoffatoms an Elektronen zurückführen.

In der *m*-Tolylborsäure-Reihe wurden ähnliche Synthesen durchgeführt. 3-Carboxy-phenylborsäure XVII, aus *m*-Tolylborsäure durch Oxydation mit alkalischer Kaliumpermanganatlösung dargestellt (3), ergab beim Nitrieren 3-Carboxy-5-nitro-phenylborsäure XVIII. Die Carboxylgruppe wurde zu Carbonsäureester, -chlorid und -amid umgewandelt und zum Amin abgebaut, ohne dass die Dihydroxyborgruppe während der Umsetzungen abgespalten wurde. Das bei dem Abbau gewonnenen Nitroamin XXIX wurde katalytisch zu dem zugehörigen Diamin XXX hydriert.

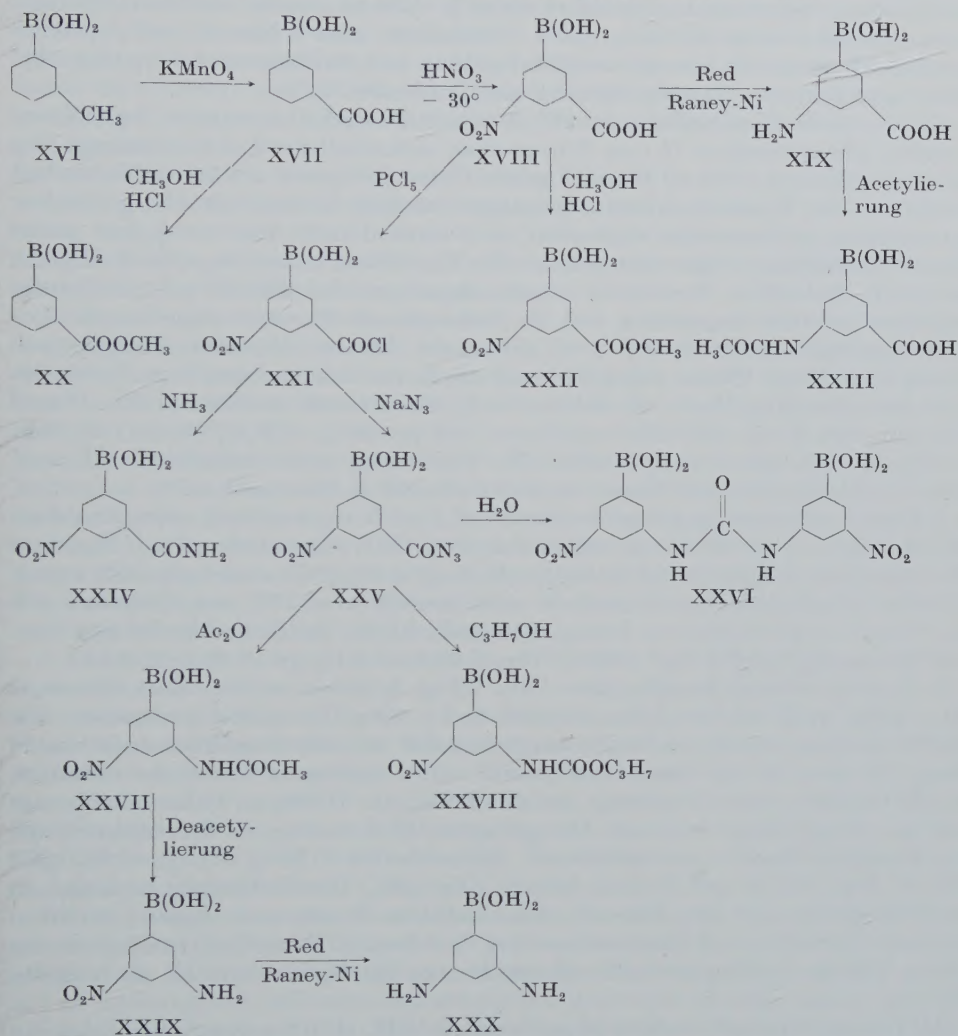
Nach den Analysen enthielten einige Arylborsäurederivate Kristallwasser, andere

* I. *Arkiv Kemi* 10 (1957), 473.

Reaktionsschema der Synthesen in der *p*-Tolylborsäure-Reihe



kamen in ihrer anhydridischen Form vor. Auch wenn die Dihydroxyborgruppe mehr oder weniger entwässert vorlag, ist sie im Reaktionsschema aus Gründen der Anschaulichkeit einheitlich bezeichnet worden.

Reaktionsschema der Synthesen in der *m*-Tolylborsäure-Reihe


Experimenteller Teil

 A. Synthesen in der *p*-Tolylborsäure-Reihe

3-Nitro-4-methyl-phenylborsäure II. 25 ml rauchende Salpetersäure (Dichte 1,50) wurden auf -42°C abgekühlt. Unter kräftigem Umrühren wurden 5 g feingepulverte *p*-Tolylborsäure I portionsweise zugesetzt, wobei die Temperatur immer bei -45°C gehalten wurde. Der Zusatz nahm etwa 5 Min. in Anspruch. Nach weiteren 5 Min. wurde der gebildete Kristallbrei auf 400 ml Eiswasser gegossen und ein paar Stunden in den Kühlschrank gestellt. Der Niederschlag wurde abgesaugt, mit etwas Eis-

wasser gewaschen und in 200 ml kalter 1 *N* Natronlauge gelöst. Die bei der Nitrierung entstandenen Nitrotoluole, die dabei nicht in Lösung gingen, wurden abfiltriert. Beim Ansäuern unter Kühlung mit 6 *N* Salzsäure kam 3-Nitro-4-methyl-phenylborsäure II heraus. Sie liess sich aus Nitromethan umkristallisieren. Schmp. 260–264° (Bean und Johnson (1) geben 260–264° an). Ausbeute: 61 %.

3-Nitro-4-carboxy-phenylborsäure III. In einem 1-Literkolben wurden 6 g 3-Nitro-4-methyl-phenylborsäure II (aus Nitromethan umkristallisiert) in Natronlauge (3 g Natriumhydroxyd + 250 ml Wasser) gelöst. Unter Erwärmen auf 50° im Wasserbad wurde eine bei Zimmertemperatur gesättigte wässrige Lösung von 12,2 g Kaliumpermanganat portionsweise zugegeben, und zwar derart, dass vor jedem neuen Zusatz Entfärbung abgewartet wurde. Die Oxydation nahm ungefähr 5 Tage in Anspruch. Gebildeter Braunstein wurde abgesaugt, das Filtrat mit verdünnter Salzsäure schwach angesäuert und im Vakuum zur Trockne eingedampft. Der Rückstand wurde dreimal mit je 25 ml kaltem abs. Äthanol extrahiert, zum gesamten Filtrat noch 10 ml Wasser zugegeben und wieder im Vakuum eingengt. Es hinterblieb eine schmierige Masse, die sich aus wenig Wasser umkristallisieren liess. Weisse Kristalle von Zersp. 220–230°. Ausbeute: 5,6 g = 80%. $C_7H_5O_6NB$ (211,0): Ber. C 39,9; H 2,87; gef. C 40,0; H 2,55. Die Verbindung ist leichtlöslich in Äthanol, löslich in Äthylacetat und Wasser und schwerlöslich in Nitromethan.

3-Nitro-4-carbomethoxy-phenylborsäure IV. 0,5 g 3-Nitro-4-carboxy-phenylborsäure III wurden in 3 ml Methanol, enthaltend 10% Chlorwasserstoff, gelöst. Man liess die Lösung eine Stunde bei 60° stehen, wonach sie in 20 ml Eiswasser gegossen wurde. Es fielen allmählich weisse Kristalle in einer Ausbeute von 42% aus. Sie liessen sich aus Wasser umkristallisieren. Schmp. 242–244°. Leicht löslich in Alkohol und heissem Wasser. $C_8H_8O_6NB.H_2O$ (243,0): Ber. C 39,5; H 4,15; gef. C 39,2; H 4,06.

3-Amino-4-carboxy-phenylborsäure VII. 1,2 g 3-Nitro-4-carboxy-phenylborsäure III wurden in 15 ml abs. Äthanol gelöst und ~ 0,5 g Raney-Nickel zugesetzt. Die Nitroverbindung wurde im Laufe von 3 Stunden mit der theoretisch berechneten Menge Wasserstoff bei Zimmertemperatur unter kräftigem Umrühren reduziert. Der Katalysator wurde abgesaugt und das Filtrat zur Hälfte im Vakuum eingengt und mit 20 ml Wasser versetzt. Der gelbgrüne Niederschlag wurde abfiltriert und aus Propanol-Wasser umkristallisiert. Rohausbeute: 0,75 g = 60%. $C_7H_8O_4NB$ (181,0): Ber. N 7,74; gef. N 7,81. Schmp. über 360°. Die Verbindung ist löslich in verd. Essigsäure und abs. Äthanol, etwas löslich in Wasser und unlöslich in Nitromethan, Acetonitril, 1,2-Dichloräthan und Cyclohexan. Beim Umkristallisieren des Amins VII aus verdünnter Salzsäure erhält man das *Hydrochlorid* IX als hellgelbe Nadeln.

2,6-Dibrom-3-amino-4-carboxy-phenylborsäure VIII. A. 0,5 g gepulverte 3-Amino-4-carboxy-phenylborsäure wurden in 15 ml Eisessig suspendiert. Unter kräftigem Umschütteln werden portionsweise 0,55 g Brom, Molverhältnis 1 : 1, gelöst in 5 ml Eisessig, zugegeben. Das Gemisch wurde 3 Stunden lang bei Zimmertemperatur geschüttelt. Die Lösung wurde danach auf 2 ml eingengt, mit 40 ml Wasser verdünnt und über Nacht in den Kühlschrank gestellt. Das Rohprodukt wurde abgesaugt, bei 50°C getrocknet und aus Wasser umkristallisiert. Goldglänzende, blättrige Kristalle. Ausbeute: 0,5 g. $C_7H_4O_3NB_2Br_2$ (anhydrisch, 320,8): Ber. N 4,37; gef. N 4,50. Schmp. über 300°. Die Verbindung ist leichtlöslich in Äthanol, löslich in verd. Essigsäure und Wasser und schwerlöslich in Nitromethan und Eisessig. Es konnte kein Monobromderivat nachgewiesen werden.

B. 0,1 g Hydrochlorid der 3-Amino-4-carboxy-phenylborsäure wurden in 5 ml

Eisessig suspendiert und 0,16 g Brom, Molverhältnis 1 : 2, gelöst in 1 ml Eisessig, zugefügt. Das Gemisch wurde unter Umrühren 2 Stunden im Wasserbad auf 50°C erwärmt. Der Eisessig wurde im Vakuum völlig abdestilliert, der Rückstand in kochendem Wasser aufgelöst und mit $N/2$ Natronlauge neutralisiert. Die Lösung wurde heiss filtriert und gekühlt, der Niederschlag abgesaugt und aus Wasser umkristallisiert. Ausbeute: 0,1 g = 64%. Goldglänzende, blättrige Kristalle, identisch mit denjenigen bei A.

Konstitutionsbeweis. 50 mg Bromverbindung VIII wurde in heissem Wasser gelöst und 50 mg Silbernitrat in 3 ml Wasser zugegeben. Es fiel sofort ein weisser Niederschlag aus, der weder Silber noch Bor enthielt. Er wurde aus 50%igem Äthanol umkristallisiert. Schmp. 234–235°. Mischschmelzpunkt mit 3,5-Dibrom-antranilsäure (Schmp. 235°) ergab keine Depression.

3-Nitro-phenylborsäure-4-carbonsäureamid V. 1,0 g feingepulverte 3-Nitro-4-carboxy-phenylborsäure III wurden innig mit 2,0 g Phosphorpentachlorid gemischt und auf dem Wasserbad in einem Kolben mit Rückflusskühler und Chlorkalzium-Rohr 90 Min. erwärmt. Dabei entstand allmählich eine klebrige Masse. Nach dem Abkühlen wurde das Phosphoroxychlorid im Vakuum auf dem Wasserbad abdestilliert. Die Trockensubstanz wurde unter Eiskühlung portionsweise in 3 ml konz. Ammoniak gelöst. Nach etwa 10 Min. Stehenlassen wurde die Lösung mit 3 ml Wasser verdünnt, unter Eiskühlung mit konz. Salzsäure schwach angesäuert und über Nacht in den Kühltank gestellt. Das Rohprodukt wurde aus Wasser umkristallisiert. Zersp. 252°. Ausbeute: 0,45 g = 50%. $C_7H_5O_5N_2B$ (210,0): Ber. N 13,3; gef. N 13,2. Die Verbindung ist löslich in Nitromethan, Wasser, abs. Äthanol und Acetonitril und unlöslich in Benzol und 1,2-Dichloräthan.

3-Amino-phenylborsäure-4-carbonsäureamid X. 1,0 g der obigen Nitroverbindung V wurden in 15 ml abs. Äthanol gelöst und ~ 0,5 g Raney-Nickel zugesetzt. Die Nitroverbindung wurde im Laufe von 2 Stunden mit der theoretisch berechneten Menge Wasserstoffgas reduziert. Der Katalysator wurde abfiltriert und das Filtrat mit 5 ml 5 N Salzsäure verdünnt. Die Lösung wurde im Vakuum auf 5 ml eingedampft. Der Rest wurde in den Kühltank gestellt, das ausgefallene Hydrochlorid abgesaugt und aus 1 N Salzsäure umkristallisiert. Grosse, farblose Platten vom Schmelzpunkt über 360°C. Für das Monohydrat des Hydrochlorids ergab eine Analyse folgende Werte: $C_7H_{12}O_4N_2ClB$ (234,5): Ber. N 11,9; gef. N 11,7.

3-Nitro-phenylborsäure-4-carbonsäureazid XI. Aus 0,5 g feingepulverter 3-Nitro-4-carboxy-phenylborsäure III und 1,0 g Phosphorpentachlorid wurde, wie oben beschrieben, das Carbonsäurechlorid dargestellt. Das Rohprodukt, 0,49 g, wurde in 6 ml Aceton gelöst und unter Eiskühlung eine gesättigte wässrige Lösung von 0,18 g Natriumazid zugetropft. Man liess die Mischung 15 Min. im Eisbad stehen, filtrierte das Natriumchlorid ab, und wusch mit 2 ml Aceton. Das Filtrat wurde ohne Erwärmen im Vakuum bis auf 2 ml eingengt und mit 22 ml Wasser verdünnt. Beim Wasserzusatz entstand ein braungelbes Öl, das allmählich erstarrte. Nach Stehen über Nacht im Kühltank wurde der gelbe Niederschlag abgesaugt und im Vakuum getrocknet. Ausbeute: 0,29 g = 52%. Da das Carbonsäureazid XI sehr unbeständig ist, wurde auf eine Analyse verzichtet.

3-Nitro-phenylborsäure-4-carbaminsäureäthylester XIII. 0,3 g des obigen Carbonsäureazids XI wurden auf dem Wasserbad mit 3 ml abs. Äthanol unter Rückfluss 45 Min. gekocht. Die warme Lösung wurde mit 9 ml Wasser verdünnt und über Nacht in den Kühltank gestellt. Der Niederschlag wurde abgesaugt und im Vakuum getrocknet. Das Rohprodukt wurde aus Eisessig umkristallisiert. Hellgelbe

Kristalle vom Schmelzpunkt 209°. Ausbeute: 0,21 g = 66%. $C_9H_{11}O_6N_2B$ (254,0): Ber. N 11,0; gef. N 10,8. Die Verbindung ist löslich in abs. Äthanol, Eisessig, Acetonitril und Nitromethan und schwerlöslich in Wasser.

N,N'-bis-[2-Nitro-phenylborsäure-(4)]-harnstoff XII. Das Filtrat, das bei der Synthese vom Carbonsäureazid XI zurückblieb, wurde eine Stunde unter Rückfluss gekocht und über Nacht in den Kühlschrank gestellt. Der orangerote Niederschlag wurde abgesaugt und im Vakuum getrocknet. Das Rohprodukt wurde aus Wasser umkristallisiert. Ausbeute: 5–10%. Zersp. 249°. $C_{13}H_{12}O_9N_4B_2$ (389,9): Ber. N 14,4; gef. N 14,7. Die Verbindung ist löslich in Wasser und Äthanol und schwerlöslich in Nitromethan und Benzol.

3-Nitro-4-acetylamino-phenylborsäure XIV. 0,5 g 3-Nitro-phenylborsäure-4-carbonsäureazid XI wurden in 6 ml Essigsäureanhydrid suspendiert und 6 Tropfen 70%ige Phosphorsäure wurden vorsichtig zugetropft, wobei unter Erwärmung des Reaktionsgemisches sofort eine kräftige Stickstoffentwicklung anfang. Die Mischung wurde $\frac{1}{2}$ Stunde auf dem Schwefelsäurebad bei 110° erwärmt. Nach Kühlung wurde die Lösung in 25 ml Wasser gegossen und 24 Stunden lang im Kühlschrank aufbewahrt. Der Niederschlag wurde abgesaugt, im Vakuum getrocknet und aus Nitromethan umkristallisiert. Ausbeute: 0,26 g = 55%. Zersp. 246°. $C_8H_9O_5N_2B$ (224,0): Ber. N 12,5; gef. N 12,5. Die Verbindung ist leichtlöslich in Äthanol, Acetonitril und Dioxan, löslich in Nitromethan und schwerlöslich in Benzol, 1,2-Dichloräthan und Wasser.

3-Nitro-4-amino-phenylborsäure XV. 1,4 g 3-Nitro-4-acetylamino-phenylborsäure XIV wurden mit 100 ml *N*/2 Schwefelsäure 1 Stunde lang unter Rückfluss gekocht, oder so lange, bis sich alles gelöst hatte. Die warme Lösung wurde mit 5 *N* Natronlauge neutralisiert und über Nacht in den Kühlschrank gestellt. Gebildetes *o*-Nitroanilin wurde zweimal mit je 5 ml Benzol extrahiert und der Rückstand aus Wasser umkristallisiert. Ausbeute: 0,72 g = 64%. Zersp. 224°. $C_6H_7O_4N_2B \cdot 1/2 H_2O$ (191,0): Ber. N 14,7; gef. N 14,7. Die Verbindung ist leichtlöslich in Äthanol und Aceton, löslich in Wasser, Nitromethan und Acetonitril und unlöslich in Benzol.

B. Synthesen in der *m*-Tolylborsäure-Reihe

3-Carboxy-phenylborsäure XVII wurde durch Oxydation von *m*-Tolylborsäure XVI nach Michaelis (3) mit alkalischer Kaliumpermanganat-Lösung dargestellt.

3-Carboxy-5-nitro-phenylborsäure XVIII. 5 g fein gepulverte 3-Carboxy-phenylborsäure wurden bei –30° portionsweise unter Umrühren zu 25 ml rauchender Salpetersäure (Dichte 1,50, hellgelb) gesetzt. Nach dem Zusatz liess man die Suspension bei derselben Temperatur eine Stunde unter Umrühren stehen, wonach sie in 150 ml Eiswasser gegossen wurde. Der gebildete Niederschlag wurde nach zweistündigem Stehen in der Kälte abfiltriert, mit etwas Eiswasser gewaschen und aus Wasser umkristallisiert. Blätterförmige blassgelbe Kristalle, ein Mol. Kristallwasser enthaltend, vom Zersp. 235–240°. Leicht löslich in Alkohol und in heissem Wasser, wenig löslich in kaltem. Schwer löslich in Benzol. Ausbeute: 4,2 g oder 61%. (Gef. C 36,9; H 3,48; ber. für $C_7H_6O_6NB \cdot H_2O$ (229,0) C 36,7; H 3,52). Man erhält die wasserfreie Säure beim Umkristallisieren aus Eisessig. (Gef. C 39,8; H 2,81; ber. für $C_7H_6O_6NB$ (211,0) C 39,9; H 2,87.)

Konstitutionsbeweis. 0,40 g Nitroverbindung XVIII wurden in 10 ml siedender wässriger 0,5 *M* Silbernitrat-Lösung gelöst und 10 Min. unter Rückfluss gekocht. Beim Erkalten fiel ein weisser Niederschlag aus. Er wurde abfiltriert und wieder

in etwas mit verdünnter Salpetersäure angesäuertem Wasser gelöst. 0,5 ml 5 N Salzsäure wurden hinzugefügt, wobei Silberchlorid ausfiel. Die Lösung wurde heiss filtriert. 0,15 g 3-Nitro-benzoesäure fiel beim Erkalten aus. Schmp. und Mischschmp. 141–142°.

3-Carbomethoxy-phenylborsäure XX. 3 g 3-Carboxy-phenylborsäure XVII wurden in 20 ml Methanol, das 10% Chlorwasserstoff enthielt, gelöst. Die Lösung wurde sich selbst eine Stunde bei 60° überlassen und wurde dann in 100 ml Eiswasser gegossen. Der Niederschlag wurde abfiltriert und im Vakuum getrocknet. Er liess sich aus Nitromethan oder Methanol-Wasser (1:4) umkristallisieren. Ausbeute 3,0 g Rohprodukt. Aus Nitromethan kam der halbanhydrische Ester heraus. Zersp. 199–202°. (Aus Nitromethan: Gef. C 55,8; H 4,68; ber. für $C_{16}H_{16}O_7B_2$ (342,0) C 56,2; H 4,68. Aus Wasser-Methanol: Gef. C 53,4; H 5,05; ber. für $C_8H_9O_4B$ (180,0) C 53,4; H 5,04).

3-Carbäthoxy-phenylborsäure wurde nach der oben beschriebenen Methode dargestellt. Sie liess sich aus Nitromethan oder Wasser-Äthanol (2:1) umkristallisieren. Zersp. 131–134°. Kuivala und Hendrickson (2) geben 136–138° an. (Gef. C 56,0; H 5,61; ber. für $C_9H_{11}O_4B$ (194,0) C 55,7; H 5,72.)

3-Carbomethoxy-5-nitro-phenylborsäure XXII. Weisse Nadeln aus Wasser-Methanol (3:1) vom Zersp. 222–224°. Rohausbeute: 88%. (Gef. C 41,9; H 3,41; ber. für $C_8H_8O_6NB$ (225,0) C 42,7; H 3,58.)

5-Nitro-phenylborsäure-3-carbonsäurechlorid XXI. 2,3 g feingepulverte 3-Carboxy-5-nitro-phenylborsäure II wurden mit 6,3 g Phosphorpentachlorid in einem Rundkolben innig gemischt und auf ein Wasserbad bei etwa 70° gesetzt. Die Mischung verflüssigte sich allmählich und nach beendeter Reaktion (eine Stunde) wurde das Phosphoroxychlorid im Vakuum abdestilliert. Das Säurechlorid XXI blieb im Kolben als eine feste hellgelbe Masse zurück. Ausbeute fast quantitativ. Es wurde nicht analysiert, sondern sofort zu weiteren Synthesen verwendet.

5-Nitro-phenylborsäure-3-carbonsäureamid XXIV. Das Säurechlorid XXI, aus 0,5 g Säure gewonnen, wurde portionsweise unter Kühlung mit Eiswasser zu 3 ml konz. Ammoniak zugesetzt. Die Lösung wurde mit 4 N Salzsäure schwach angesäuert und anschliessend heiss filtriert. Beim Erkalten fiel das Säureamid XXIV aus. Es wurde aus Alkohol-Wasser (1:10) umkristallisiert. Weisse Nadeln vom Zersp. 265–266°. Ausbeute: 79%. (Gef. N 13,27; ber. für $C_7H_7O_5N_2B$ (210,0) N 13,34.)

5-Nitro-phenylborsäure-3-carbonsäureazid XXV. Das Säurechlorid XXI, aus 2,3 g 3-Carboxy-5-Nitro-phenylborsäure XVIII erhalten, wurde in 15 ml Aceton gelöst. Unter guter Kühlung mit Eiswasser und kräftigem Schütteln wurden 0,75 g Natriumazid in 2 ml Wasser zugegeben. Man liess die Lösung 10 Min. stehen, filtrierte den Niederschlag ab und wusch mit ein paar ml Aceton nach. 60 ml Wasser wurden dem Filtrat zugegeben. Das Azid XXV fiel allmählich aus. Rechteckige blassgelbe Kristalle vom Zersp. etwa 130°. Ausbeute: 72%.

3-Carbo-n-propoxy-amino-5-nitro-phenylborsäure XXVIII. 0,3 g 5-Nitro-phenylborsäure-3-carbonsäureazid XXV wurden in 4 ml *n*-Propanol drei Viertelstunden unter Rückfluss gekocht. Die gelbe Lösung wurde mit 15 ml Wasser versetzt, wobei der Carbaminsäureester XXVIII zum Teil in Form von Öltropfen ausfiel. Die Öltropfen erstarrten beim Stehen in der Kälte. Der Niederschlag wurde in Wasser-Eisessig (15:1) umkristallisiert. Blassgelbe körnige Kristalle vom Zersp. 140–142°. Ausbeute: 67%. (Gef. C 45,0; H 4,91; N 10,4; ber. für $C_{10}H_{13}O_6N_2B$ (268,0) C 44,8; H 4,85; N 10,5.)

N,N'-bis-[3-nitro-phenylborsäure-(4)]-harnstoff XXVI. 0,6 g Azid XXV wurden

eine halbe Stunde in 30 ml Wasser unter Rückfluss gekocht. Beim Erkalten fielen orangefarbige, nadelförmige Kristalle aus, die aus Wasser-Eisessig (10:1) umkristallisiert wurden. Zersp. 325–330°. Ausbeute: 0,3 g oder 58%. Gef. C 39,4; H 3,32; N 13,81; ber. für $C_{13}H_{12}O_5N_4B_2$ (389,9) C 40,0; H 3,11; N 14,37.

3-Acetylamino-5-nitro-phenylborsäure XXVII. 2 g Azid XXV wurden vorsichtig in 16 ml Essigsäure-anhydrid gelöst. Ein ml 70%ige Phosphorsäure wurde zugegeben. Die Lösung erwärmte sich und eine schwache Gasentwicklung setzte ein. Auf einem Schwefelsäurebad wurde die Lösung langsam auf 100–110° erhitzt, wobei die Gasentwicklung bald lebhaft wurde. Nach Abklingen der Reaktion liess man die Lösung noch eine Viertelstunde bei derselben Temperatur stehen. Es ist nicht zweckmässig, grössere Mengen auf einmal umzulagern. Die Reaktion kann leicht so heftig werden, dass der ganze Inhalt aus dem Kolben hinausspritzt. Nach Erkalten wurde die Lösung in 130 ml Eiswasser gegossen. Der hellgelbe Niederschlag wurde aus Äthanol-Wasser 1:1, umkristallisiert. Zersp. 267°. Ausbeute: 1,7 g oder 87% vom Rohprodukt. Gef. C 42,4; H 4,07; N 12,4; ber. für $C_8H_9O_5N_2B$ (224,0) C 42,8; H 4,05; N 12,5.

3-Amino-5-nitro-phenylborsäure XXIX. 0,1 g 3-Acetylamino-5-nitro-phenylborsäure XXVII wurde 10 Min. mit 2 ml 4 N Salzsäure gekocht. Die Substanz löste sich und bildete eine gelbe Lösung. Sie wurde heiss mit etwa 5 N Natronlauge auf pH 5 neutralisiert. Rote, flache Nadeln, leicht löslich in sowohl Säure als Lauge, fielen aus. Sie liessen sich aus Wasser umkristallisieren. Bei etwa 200° wird die Substanz gelb und färbt sich dunkel bei 260°. Zersp. 270–275°. Ausbeute: 0,060 g oder 74%. Gef. N 15,54; ber. für $C_6H_7O_4N_2B$ (182,0) N 15,40.

Konstitutionsbeweis. 0,1 g vom Nitroamin XXIX wurde 10 Min. mit 5 ml 0,4 M Silbernitratlösung gekocht. Es fielen beim Erkalten 0,065 g *m*-Nitroanilin aus. Schmp. und Mischschmp. 111°.

3,5-Diamino-phenylborsäure XXX. 0,4 g 3-Amino-5-nitro-phenylborsäure XXIX wurden bei Zimmertemperatur in 10 ml absolutem Alkohol katalytisch hydriert. Raney-Nickel wurde als Katalysator verwendet. Nach einer Stunde war die Reaktion beendet, wobei die theoretische Wasserstoffmenge absorbiert wurde. Die durch Filtrieren vom Katalysator befreite Lösung wurde im Vakuum eingedampft. Der Rückstand wurde aus Wasser mit Zusatz von Aktivkohle umkristallisiert. Farblose Blätter. Ausbeute: 0,25 g. Schmilzt unter Wasserabgabe bei 116°, wird bald wieder fest und zersetzt sich bei 170–180° unter Verkohlen. Gef. N 14,79; ber. für $C_6H_9O_2N_2$. $\cdot 2H_2O$ (188,0) N 14,91.

3-Carboxy-5-amino-phenylborsäure XIX. 14 g 3-Carboxy-5-nitro-phenylborsäure XVIII in 125 ml absolutem Alkohol wurden mit Raney-Nickel bei Atmosphärendruck und Zimmertemperatur katalytisch hydriert. Die Reduktion war nach sechs Stunden beendet. Die theoretisch berechnete Wasserstoffmenge wurde aufgenommen. Das Raney-Nickel wurde abfiltriert und das Filtrat zur Hälfte im Vakuum eingengt. 500 ml Eiswasser wurden zugegeben, wobei sich das Amin als weisser Niederschlag abschied. Es liess sich aus Wasser umkristallisieren. Farblose, lange, verfilzte Nadeln vom Zersp. 212–214°. Ausbeute: 10 g oder 70%. Die Substanz enthält drei Mol. Kristallwasser. Gef. C 35,4; H 6,10; N 6,12; ber. für $C_7H_8O_4NB \cdot 3H_2O$ (235,0) C 35,8; H 6,00; N 5,96.

Beim Umkristallisieren des Amins XIX aus verdünnter Salzsäure kommt das *Amin-hydrochlorid* in Form von weissen Nadeln heraus. Löslich in Wasser, schwerlöslich in Alkohol und Aceton.

3-Carboxy-5-benzoylamino-phenylborsäure. 0,3 g Amin XIX in 10 ml Kalilauge gelöst, wurden in einem Reagensglas mit 0,26 g Benzoylchlorid geschüttelt. Die Lösung

wurde nach beendeter Reaktion mit verdünnter Salzsäure schwach angesäuert. Die Benzoylverbindung lässt sich aus Wasser umkristallisieren. Lange, weisse Nadeln vom Schmp. 255°. Ausbeute: 0,38 g. Die Substanz enthält zwei Mol Kristallwasser. Gef. C 52,0; H 4,98; N 4,29; ber. für $C_{14}H_{12}O_5NB \cdot 2H_2O$ (321,1) C 52,3; H 5,03; N 4,36.

3-Carboxy-5-acetylamino-phenylborsäure XXIII wurde durch Acetylierung des Amins XIX mit Essigsäureanhydrid dargestellt. Weisse Kristalle vom Zersp. 226–228°. Löslich in heissem Wasser und Eisessig. Unlöslich in Nitromethan. Gef. C 48,6; H 4,47; N 6,10; ber. für $C_9H_{10}O_5NB$ (223,0) C 48,5; H 4,52; N 6,28.

Zusammenfassung

Es werden die Darstellung und Eigenschaften weiterer Derivate der Phenylborsäuren beschrieben. Eine Reihe Reaktionen wie Nitrierung, Kernbromierung, Hydrierung, Veresterung und Curtiussehe Umlagerung sind mit den Arylborsäuren durchgeführt worden, ohne dass eine Abspaltung der Dihydroxyborgruppe eintrat.

Für die Unterstützung dieser Arbeit danken wir bestens *Hierta-Retzius' fond för vetenskaplig forskning* und *Stiftelsen Lars Hiertas minnes fond*.

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Zur Kenntnis der Arylborsäuren. III*

Bromierung der Tolylborsäuren nach Wohl-Ziegler

VON KURT TORSSELL

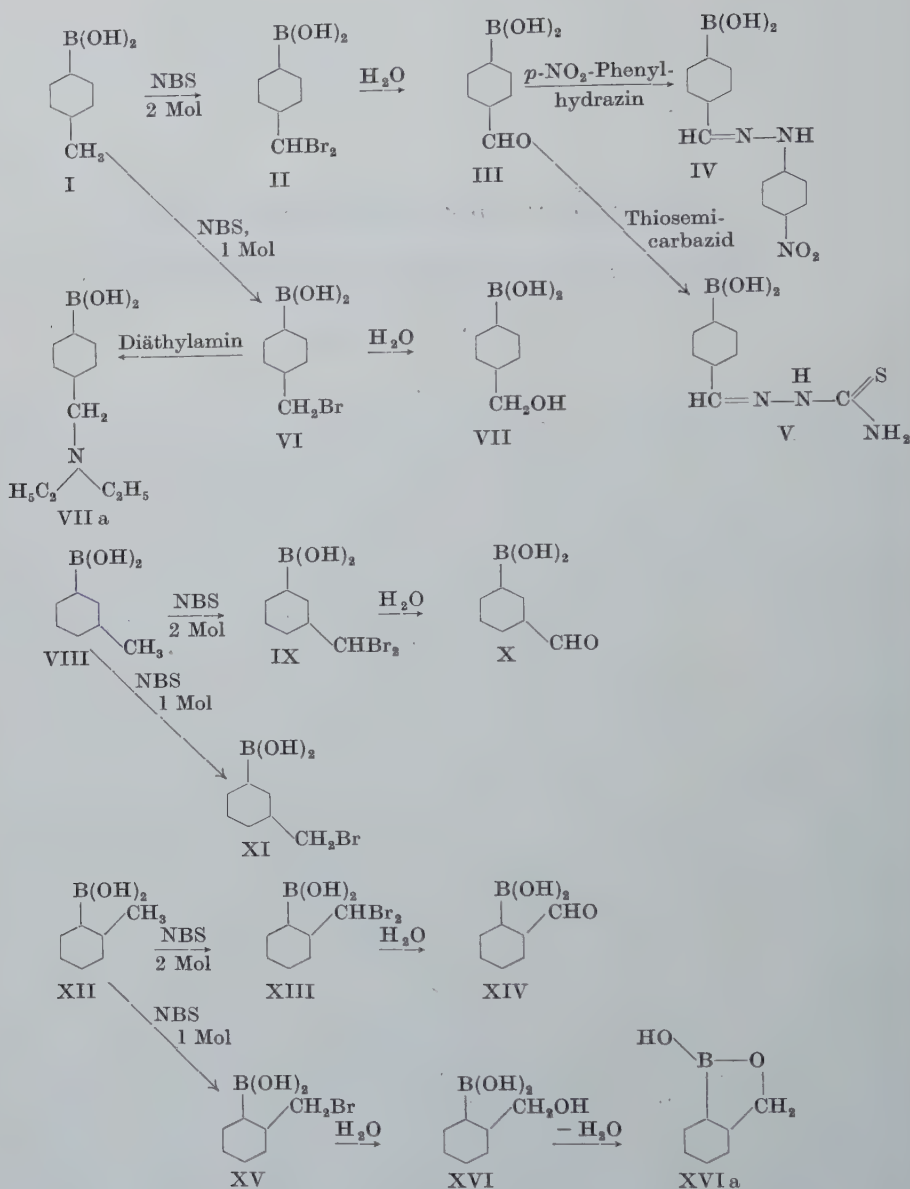
Wegen der Empfindlichkeit der B-C-Bindung gegen elektrophile Reagenzien eignet sich freies Brom im allgemeinen nicht zur Darstellung bromhaltiger Arylborsäuren. Die Dihydroxyborgruppe wird dabei gegen Brom nach einem ionischen Substitutionsmechanismus ausgetauscht. Kuivila und Mitarbeiter (1) haben erwiesen, dass die Bromolyse von chlor- und nitrosubstituierten Derivaten träger verläuft als die von Phenylborsäure selbst. Andererseits ist dann zu erwarten, dass die Tolylborsäuren noch rascher gespalten werden, was auch der Fall ist (1). Die Einführung von Substituenten, die die Elektronendichte des Kerns erniedrigen, bewirkt also eine Stabilisierung der B-C-Bindung, während ihre Stabilität herabgesetzt wird von Substituenten, die die Elektronendichte erhöhen. Das Verhalten der 3-Amino-4-carboxy-phenylborsäure gegen Brom steht ganz im Einklang mit diesen Befunden. Es findet hier in 2- und 6-Stellung eine Kernbromierung statt (2). Die Carboxygruppe und das Ammoniumion ziehen vom Kern Elektronen an, die freie Aminogruppe aber erhöht infolge mesomerer Elektronenverschiebungen die negative Ladung der Kohlenstoffatome 2 und 6 und die Bromierung wird deswegen besonders an diesen Stellen bevorzugt. Die Stabilität der B-C-Bindung gegen N-Bromsuccinimid (NBS), das sich nach einem radikalischen Mechanismus umsetzt, stützt weiterhin die Theorie eines ionischen Substitutionsvorgangs.

Es war von besonderem Interesse, ein reaktives Bromatom in die Seitenkette einzuführen, denn dadurch wird die Darstellung neuartiger bororganischer Verbindungen ermöglicht. Da eine Bromierung mit freiem Brom als ausgeschlossen erschien, wurde zunächst die Wohl-Zieglersche Reaktion probiert (3).

Die Versuche wurden in Kohlenstofftetrachlorid als Lösungsmittel nach dem üblichen Verfahren durchgeführt. Die Reaktion tritt beim Zusatz von Benzoylperoxyd als Katalysator überraschend leicht ein und vollzieht sich ziemlich rasch. Mit einem Mol NBS pro Mol Tolylborsäure erhält man die ω -Brom-tolylborsäuren in guten Ausbeuten. Da die Bromderivate in heissem Kohlenstofftetrachlorid ziemlich leicht und in kaltem nur wenig löslich sind, lassen sie sich nach beendeter Reaktion sehr einfach durch Abfiltrieren des schwerlöslichen Succinimids und nachfolgendes Kühlen des Filtrats isolieren. Die Bromverbindungen werden langsam an der Luft hydrolysiert, sind aber sonst stabil.

Die drei isomeren ω -Brom-tolylborsäuren sind dargestellt worden (VI, XI und XV). Aus ω -Brom-*o*-tolylborsäure entsteht bei der Hydrolyse *o*-Hydroxymethylphenylborsäure. Nach der Analyse liegt sie in ihrer anhydriischen Form vor, aber

* II. *Arkiv Kemi* 10 (1957), 497.



wahrscheinlich hat sich in diesem Fall unter Ringschluss und Wasseraustritt ein innerer Ester XVIa gebildet.

Wenn die Tolylborsäuren mit zwei Mol NBS umgesetzt werden, bilden sich die ω,ω -Dibrom-tolylborsäuren, die bei der Hydrolyse die entsprechenden Formyl-derivate als gut kristallisierende stabile Verbindungen ergeben. Sie setzen sich in gewöhnlicher Weise mit 4-Nitro-phenylhydrazin und Thiosemicarbazid unter Bildung von Hydrazon IV bzw. Carbazon V um.

Versuche, *o*-Carboxy-phenylborsäure aus *o*-Tolylborsäure durch Oxydation mit alkalischer Kaliumpermanganatlösung darzustellen, führten nicht zum Ziel (4). Auch unsere Versuche, diese Säure durch Oxydation des Formylderivats bzw. Umsetzung der *o*-Tolylborsäure mit drei Mol NBS zu gewinnen, waren erfolglos. Im ersten Fall konnte nur etwas Benzoesäure nachgewiesen werden. Bei der Bromierung trat teilweise Zersetzung ein, wobei kernbromierte Spaltprodukte und etwas *o*-Formylborsäure isoliert werden konnten.

Experimenteller Teil

Die *Tolylborsäuren* wurden nach der von Torssell (5) modifizierten Methode dargestellt.

ω-Brom-*p*-tolylborsäure VI. 5 g *p*-Tolylborsäure I wurden in 190 ml Kohlenstofftetrachlorid gelöst. 6,8 g feingepulvertes N-Bromsuccinimid (98%) und etwa 0,1 g frisch gefälltes Dibenzoylperoxyd wurden zugefügt. Die Mischung wurde eine Stunde auf dem Wasserbad unter Rückfluss und Feuchtigkeitausschluss gekocht. Das schwere N-Bromsuccinimid ging allmählich in das Succinimid über, das am Ende der Reaktion an der Oberfläche schwebte. Die hellgelbe Lösung wurde schnell heiss durch einen Büchnertrichter filtriert. 4,0 g rohes Succinimid (theoretisch 3,9 g) wurden zurückgewonnen. Aus dem Filtrat kam die *ω*-Brom-*p*-tolylborsäure VI fast unmittelbar als weisse Nadeln heraus. Rohausbeute: 7,1 g oder 90%. Nach Umkristallisieren aus trockenem Kohlenstofftetrachlorid schmilzt die Substanz bei 165–168°. Sie muss unter Ausschluss von feuchter Luft aufbewahrt werden. Gef. C 39,16; H 2,84; Br 37,1; ber. für $C_7H_8O_2Br$ (214,9) C 39,12; H 3,75; Br 37,2.

ω-Brom-*m*-tolylborsäure XI. Das *m*-Derivat wurde nach der oben beschriebenen Methode dargestellt. Aus 1,5 g *m*-Tolylborsäure VIII, 2,1 g N-Bromsuccinimid und etwas Dibenzoylperoxyd in 40 ml Kohlenstofftetrachlorid wurden 1,6 g *ω*-Brom-*m*-tolylborsäure XI gewonnen. Die Reaktion war nach 35 Min. beendet. Zur Analyse wurde die Substanz aus trockenem Nitromethan umkristallisiert. Schmp. 214–216°. Gef. Br 40,1; ber. für C_7H_8OBr (anhydriisch, 196,9) Br 40,6.

ω-Brom-*o*-tolylborsäure XV wurde aus *o*-Tolylborsäure XII, 1,5 g, N-Bromsuccinimid, 2,1 g, und 0,1 g Dibenzoylperoxyd in 35 ml Kohlenstofftetrachlorid dargestellt. Ausbeute: 1,7 g oder 79%. Liess sich aus Kohlenstofftetrachlorid umkristallisieren. Sintert bei 142° und schmilzt bei 148°. Gef. C 42,08; H 3,28; ber. für C_7H_8OBr (anhydriisch, 196,9) C 42,70; H 3,08.

o-Hydroxymethyl-phenylborsäure XVI. 0,4 g *ω*-Brom-*o*-tolylborsäure wurden in 6 ml Wasser 10 Min. gekocht. Es fielen beim Erkalten weisse, lange, flache Nadeln aus, die aus Wasser umkristallisiert wurden. Schmp. 97–98°. Ausbeute: 0,1 g. Gef. C 62,83; H 5,27; ber. für $C_7H_7O_2B$ (anhydriisch, 134,0) C 62,74; H 5,27.

Es wurde auch versucht, *p*-Hydroxymethyl-phenylborsäure VII durch Umsetzung von *ω*-Brom-*p*-tolylborsäure VI mit siedendem Wasser darzustellen. Das Produkt war sehr leicht löslich in Wasser und liess sich nicht daraus umkristallisieren. Man kann es auch nicht aus anderen Lösungsmitteln reinigen. Bei der Hydrolyse wurde ein wenig *p*-Formyl-phenylborsäure III isoliert. Es bildet sich also bei der Monobromierung auch etwas *ω,ω*-Dibrom-*p*-tolylborsäure II.

Die Versuche, die entsprechende *ω*-Aminoverbindung rein zu isolieren, scheiterten. Eine Lösung von 0,5 g *ω*-Brom-*p*-tolylborsäure VI in 12 ml absolutem Alkohol, enthaltend 10% Ammoniak, wurde über Nacht sich selbst überlassen. Das Äthanol

wurde im Vakuum abdestilliert. Der Rückstand war in Wasser schwerlöslich, in verdünnter Säure aber sehr leicht löslich. Beim Umkristallisieren aus wenig 1 N Salzsäure wurde etwa 0,1 g eines uneinheitlichen Produktes isoliert. Aus dem Filtrat fiel beim Neutralisieren wieder das Amin aus. Es liess sich durch Umkristallisieren nicht reinigen.

ω-Diäthylamino-p-tolylborsäure VIIa konnte auch nicht in reinem Zustand isoliert werden. Das Bromid wurde mit Diäthylamin (Molverhältnis 1:2) in äthanolischer Lösung umgesetzt. Beim Einengen des Äthanols blieb ein Öl zurück, das beim Zusatz von Wasser in den festen Zustand überging. Es ist schwerlöslich in Wasser. Sehr leicht löslich in Säuren.

ω,ω-Dibrom-p-tolylborsäure II. 2,5 g *p*-Tolylborsäure I wurden in 90 ml Kohlenstofftetrachlorid aufgelöst, 6,8 g N-Bromsuccinimid (98%) und etwas frisch gefälltes Dibenzoylperoxyd wurden zugesetzt. Nach einstündigem Kochen unter Rückfluss und Feuchtigkeitausschluss hatte sich das N-Bromsuccinimid vollständig umgesetzt. Es wurde heiss filtriert. Sofort schieden sich aus dem Filtrat schwere Kristalle ab. Rohausbeute: 4,5 g. Das Dibromid zersetzt sich teilweise infolge Hydrolyse beim Umkristallisieren (Kohlenstofftetrachlorid oder Toluol). Zersp. 160–170°. Die Analyse ergab einen etwas zu niedrigen Bromgehalt.

p-Formyl-phenylborsäure III. 0,5 g *ω,ω*-Dibrom-*p*-tolylborsäure II wurden 10 Min. in 5 ml mit Schwefeldioxyd gesättigtem Wasser gekocht. Beim Erkalten fielen weisse Stäbchen des Aldehyds aus, die aus Wasser umkristallisiert wurden. Die Substanz sintert bei 230–240° zu einer glasartigen Masse. Ausbeute: 0,16 g. Gef. C 52,12; H 4,86; ber. für $C_7H_7O_3B \cdot 1/2 H_2O$ (159,0) C 52,88; H 5,07.

p-Formyl-phenylborsäure-p-nitrophenylhydrazon IV. Zu einer warmen Lösung von 0,07 g *p*-Nitrophenylhydrazin in 6 ml 50%igem Äthanol wurden 0,07 g fester *p*-Formyl-phenylborsäure III gesetzt. Man liess die Lösung eine Stunde bei 80° stehen. Beim Erkalten schieden sich rote Nadeln ab. Sie wurden abfiltriert, mit 40%igem Äthanol gewaschen und aus 70%igem Methanol umkristallisiert. Zersp. etwa 280°. Schwerlöslich in Wasser, Toluol und Nitromethan. Leicht löslich in Alkohol. Gef. C 54,53; H 4,72; N 14,21; ber. für $C_{13}H_{12}O_4N_3B$ (285,1) C 54,75; H 4,25; N 14,74.

p-Formyl-phenylborsäure-thiosemicarbazon V. Zu einer warmen Lösung von 0,35 g *p*-Formyl-phenylborsäure III in 15 ml 1 N Essigsäure wurden 0,35 g Thiosemicarbazid in 6 ml Wasser zugesetzt. Das Carbazon fiel schnell als weisser Niederschlag aus. Ausbeute: 0,45 g. Liess sich aus Wasser-Äthanol (3:1) umkristallisieren. Zersp. 204–205°. Leichtlöslich in Aceton. Gef. C 41,16; H 4,66; ber. für $C_8H_{10}O_2N_3SB \cdot 1/2 H_2O$ (232,1) C 41,40; H 4,76.

ω,ω-Dibrom-o-tolylborsäure XIII. Eine Mischung von 1,5 g *o*-Tolylborsäure XII, 4,2 g N-Bromsuccinimid (Molverhältnis 1:2) und 0,1 g Dibenzoylperoxyd in 35 ml Kohlenstofftetrachlorid wurden anderthalb Stunden unter Rückfluss und Feuchtigkeitausschluss gekocht. Das N-Bromsuccinimid hatte sich vollständig umgesetzt. Das an der Oberfläche schwimmende Succinimid wurde abfiltriert und das Filtrat im Vakuum eingengt. Der Rückstand wurde aus Cyclohexan umkristallisiert. Ausbeute: 56%. Die Substanz sintert bei 155° und schmilzt bei 163°. Sie hydrolysiert sich langsam an der Luft. Der gefundene Kohlenstoffwert ist deshalb etwas höher als berechnet. Gef. C 31,64; H 2,23; ber. für $C_7H_5OBr_2B$ (anhydrisch, 275,8) C 30,49; H 1,84.

o-Formyl-phenylborsäure XIV wurde ähnlich wie die *p*-Verbindung dargestellt. Sie wurde aus Nitromethan umkristallisiert. Sintert bei 115° und schmilzt bei 123°. Schwerlöslich in Kohlenstofftetrachlorid und Cyclohexan. Etwas löslich in Toluol.

Gut löslich in heissem Wasser, weniger in kaltem. Gef. C 55,82; H 4,92; ber. für $C_7H_7O_3B$ (150,0) C 56,04; H 4,71.

ω,ω -Dibrom-*m*-tolylborsäure IX ähnelt in ihren Eigenschaften den anderen Isomeren. Sie wurde nach der für die entsprechende *p*-Verbindung beschriebenen Methode dargestellt. Ausbeute von Rohprodukt: 69 %. Zum Umkristallisieren eignet sich Nitromethan. Zersp. 160–165°.

m-Formyl-phenylborsäure X wurde in 47%iger Ausbeute aus Dibromid und Wasser gewonnen. Sie wurde aus Wasser umkristallisiert. Schmilzt bei 112° unter Wasserabgabe. Sie wird bald wieder fest und schmilzt dann erst bei etwa 180°. Gef. C 55,10; H 4,50; ber. für $C_7H_7O_3B$ (150,0) C 56,04; H 4,71.

Versuche, *o*-Carboxy-phenylborsäure darzustellen. a. 1,5 g *o*-Tolylborsäure XII wurden mit 6,3 g N-Bromsuccinimid (Molverhältnis 1:3) und 0,2 g Dibenzoylperoxyd in 35 ml Kohlenstofftetrachlorid unter Rückfluss 6 Stunden lang gekocht. Die Lösung wurde allmählich dunkel. Das Peroxyd wurde in kleinen Portionen während der Umsetzung zugegeben. Die Lösung wurde filtriert und im Vakuum eingeeengt. Ein braunes Öl blieb zurück, das nur teilweise beim Kochen mit Wasser in Lösung ging. Der Rückstand bestand wahrscheinlich aus einem kernbromierten Produkt. Aus der Wasserlösung schied sich beim Erkalten 0,7 g *o*-Formylborsäure XIV ab. *o*-Carboxy-phenylborsäure konnte nicht nachgewiesen werden.

b. Zu 0,45 g *o*-Formyl-phenylborsäure und 0,2 g Natriumhydroxyd in 15 ml Wasser wurde eine Lösung von 0,30 g Kaliumpermanganat in 10 ml Wasser gegeben. Nach kurzer Zeit begann Mangandioxyd auszufallen. Die Reaktion war nach einem Tag beendet. Das Mangandioxyd wurde abfiltriert und aus der Lösung kam beim schwachen Ansäuern mit Salzsäure ein weisser Niederschlag heraus, der als Benzoesäure identifiziert wurde.

Zusammenfassung

Die Tolylborsäuren setzen sich mit einem Mol NBS unter Bildung von ω -Monobromderivaten und mit zwei Mol NBS unter Bildung von ω,ω -Dibromderivaten um. Bei der Hydrolyse der Bromverbindungen entstehen die entsprechenden Hydroxymethyl- und Formyl-phenylborsäuren.

Für die ökonomische Unterstützung dieser Arbeit bin ich *Stiftelsen Lars Hiertas Minne* und *Hierta-Retzius' fond för vetenskaplig forskning* zu besonderem Dank verpflichtet.

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Zur Kenntnis der Arylborsäuren. IV*

Nitrierung einiger Arylborsäuren und Eigenschaften der Bor-Kohlenstoff-Bindung

Von KURT TORSELL

Einleitung

Nitrierung von Arylborsäuren sind in einigen Fällen mit gutem Erfolg durchgeführt worden. Die drei isomeren Mononitro-phenylborsäuren lassen sich durch vorsichtiges Nitrieren von Phenylborsäure darstellen (1, 2) und aus *p*-Brom-phenylborsäure (3) und *p*-Tolylborsäure (3, 4) erhält man 3-Nitro-4-brom- bzw. 3-Nitro-4-methyl-phenylborsäure. 3- und 4-Carboxy-phenylborsäure ergeben in guter Ausbeute 3-Carboxy-5-nitro- bzw. 4-Carboxy-2-nitro-phenylborsäure (4, 5). Es wird nun das Verhalten weiterer Arylborsäurederivate beim Nitrieren untersucht. Daraus und aus den in den früheren Mitteilungen beschriebenen Umsetzungen und charakteristischen Reaktionen sind Schlüsse über den Mechanismus der Dihydroxyborgruppenabspaltung und über die allgemeine Stabilität verschiedener Arylborsäuren gezogen worden.

Besprechung der Versuche

o- und *m*-Tolylborsäure erwiesen sich empfindlicher gegenüber Salpetersäure als das *p*-Isomer. In rauchender Salpetersäure bei -40° bis -45° und in Essigsäureanhydrid als Lösungsmittel führte Nitrieren stets zur Zersetzung, wobei sich Nitrotoluole bildeten. Dagegen gelang die Nitrierung von *o*-Tolylborsäure I in einer breiigen Mischung von Schwefelsäure und Salpetersäure bei -40° bis -45° . Dabei entstand ein Dinitroderivat, 2-Methyl-3,5-dinitro-phenylborsäure II, das sich durch katalytische Hydrierung in das entsprechende Diamin III überführen liess.

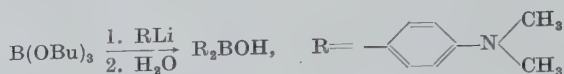
m-Acetylamino-phenylborsäure V ergab mit rauchender Salpetersäure in überraschend guter Ausbeute 6-Nitro-3-acetylamino-phenylborsäure VI. Zwei andere Isomeren, 2- und 4-Nitro-3-acetylamino-phenylborsäure VIII und IX, wurden nachgewiesen, deren Ausbeute durch Nitrieren in Essigsäureanhydrid beträchtlich gesteigert werden konnte.

α -Naphthylborsäure und Thienylborsäure ergaben beim Nitrieren nur schmierige Zersetzungsprodukte. *p*-Methoxy-phenylborsäure lieferte 2,4-Dinitroanisol.

p-Dimethylamino-phenylborsäure wurde dargestellt, um ihre physiologischen Wirkungen zu studieren. Da Magnesium nicht mit *p*-Brom-dimethylanilin reagiert, wurde die Li-organische Verbindung dargestellt und mit Tributylborat zur Reaktion

* III. *Arkiv Kemi* 10 (1957), 507.

gebracht. Trotz tiefer Temperatur und Überschuss von Tributylborat bildet sich hauptsächlich Diarylborsäure.

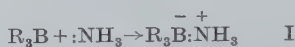


Sie ist unbeständig und zersetzt sich an der Luft unter Bildung von *p*-Dimethylanilin und *p*-Dimethylamino-phenylborsäure XII. Auch XII zerfällt langsam, ist aber stabiler als *p*-Amino-phenylborsäure.

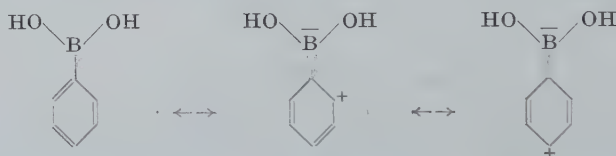
Dirigierende Wirkung der Dihydroxyborgruppe

In Tabelle I sind die Resultate der bisher durchgeführten Nitrierungen zusammengestellt. Man sieht daraus, dass die Dihydroxyborgruppe in Salpetersäure als Lösungsmittel in *meta*-Stellung und in Essigsäureanhydrid in *ortho-para*-Stellung dirigiert. Dieses Verhalten lässt sich durch folgende Überlegungen erklären.

Die dreiwertigen Borverbindungen sind im Lewisschen Sinne Säuren. Das Bor strebt, seine Oktettlücke unter Bildung eines Dipols I oder eines Anions II aufzufüllen.



Eine Komplettierung des Oktettes kann auch durch mesomere Elektronenverschiebungen zustande kommen.



Die Dihydroxyborgruppe setzt also die Elektronendichte des Kerns herab, und zwar derart, dass die *ortho-para*-Stellung mehr deaktiviert wird als die *meta*-Stellung. Der neu eintretende Substituent wird demgemäss nach der *meta*-Stellung gelenkt.

Da der Kohlenstoff elektronegativer ist als das Bor werden die Bindungselektronen etwas zum Kohlenstoff hinübergezogen. Weiterhin ist es möglich, dass das freie Dublett am Sauerstoff in die Oktettlücke des Bors hineingezogen werden kann. Diese beiden Effekte wirken dem ersten entgegen und erhöhen die Elektronendichte des Kerns. Sie scheinen hier aber ohne Bedeutung zu sein.

Nitrieren von substituierten Phenylborsäurederivaten gibt Auskunft über die relative Stärke der dirigierende Wirkung. Es hat sich dabei herausgestellt, dass *p*-Carboxy-phenylborsäure in 2-Stellung substituiert wird. 2-Nitro-4-carboxy-phenylborsäure wurde in 67%iger Ausbeute isoliert (5). Die Bildung vom 3-Isomer lässt sich natürlich nicht ausschliessen, sie konnte aber nicht nachgewiesen werden. Es geht daraus hervor, dass die *meta*-dirigierende Wirkung der Carboxygruppe viel grösser ist als die der Dihydroxyborgruppe.

3-Acetylamino-phenylborsäure V ergab beim Nitrieren 69% 6-Nitro-3-acetylamino-

Tab. 1. Nitrieren von Arylborsäuren.

Nitrieren von	Medium	Temp. °C	Produkte	Aus- beute %	Literatur
Phenylborsäure	r. HNO ₃	-15 bis -9	<i>m</i> -NO ₂ -Phenylborsäure	70	(2); Die-
Tolylborsäure	»	-30 bis -28	<i>o</i> -NO ₂ -Phenylborsäure	10	se Arb.
»	»	-45 bis -40	3-NO ₂ -4-CH ₃ -Phenylborsäure	75	(3)
Br-Phenylborsäure	»	-15	»	61	(4)
COOH-Phenylbors.	»	-30 bis -25	3-NO ₂ -4-Br-Phenylborsäure	45-50	(3)
Tolylborsäure	r. HNO ₃ ; Ac ₂ O/ r. HNO ₃	-40; -20	2-NO ₂ -4-COOH-Phenylbors.	67	(5)
NHAc-Phenylbors.	r. HNO ₃	-45 bis -40	Zersetzungsprodukte	—	Diese Arb.
COOH-Phenylbors.	»	-30	6-NO ₂ -3-NHAc-Phenylbors.	69	» »
Tolylborsäure	»	-40; -20	2-NO ₂ -3-NHAc-Phenylbors.	9	» »
CH ₃ O-Phenylbors.	r. HNO ₃ ; Ac ₂ O/	»	4-NO ₂ -3-NHAc-Phenylbors.	61	(4)
Thienylborsäure	r. HNO ₃	»	5-NO ₂ -3-COOH-Phenylbors.	—	Diese Arb.
Naphtylborsäure	»	»	Zersetzungsprodukte	71	» » (3)
Tolylborsäure	H ₂ SO ₄ /r. HNO ₃	-45 bis -40	2,4-NO ₂ -Anisol	—	» »
Phenylborsäure	Ac ₂ O/r. HNO ₃	-15 bis 20	Zersetzungsprodukte	—	» »
NHAc-Phenylbors.	»	-15 bis 0	2-CH ₃ -3,5-NO ₂ -Phenylbors.	75	» »
			<i>o</i> -NO ₂ -Phenylborsäure	62	» »
			<i>p</i> -NO ₂ -Phenylborsäure	8	» »
			6-NO ₂ -3-NHAc-Phenylbors.	12	» »
			2-NO ₂ -3-NHAc-Phenylbors.	26	» »
			4-NO ₂ -3-NHAc-Phenylbors.		

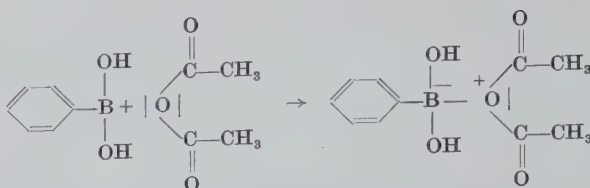
r. HNO₃ = rauchende Salpetersäure; Ac₂O/r. HNO₃ = rauchende Salpetersäure in Essigsäureanhydrid als Lösungsmittel.

phenylborsäure VI und za. 9% von den 2- und 4-Isomeren. 5-Nitroderivat konnte nicht nachgewiesen werden. Die *ortho-para*-dirigierende Wirkung der Acetylaminogruppe hebt also die *meta*-dirigierende Wirkung der Dihydroxyborgruppe auf.

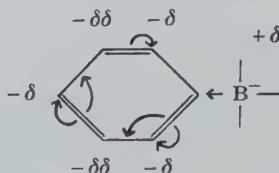
Bettman, Branch und Yabroff (6) haben für *m*- und *p*-Carboxy-phenylborsäure in 25%iger Alkohollösung bei 25° die Dissoziationskonstanten $2,22 \times 10^{-5}$ bzw. $3,06 \times 10^{-5}$ (Carboxygruppe) gefunden. Zum Vergleich wurde die Dissoziationskonstante der Benzoesäure in demselben Lösungsmittel gemessen; $K_s = 2,29 \times 10^{-5}$. Wie erwartet ist die Dihydroxybor-Verbindung eine stärkere Säure als Benzoesäure selbst. $\Delta pK = \sigma = +0,13$. Die *m*-ständige Dihydroxyborgruppe hat erwartungsgemäss wenig Einfluss auf die Säurestärke, $\Delta pK = \sigma = -0,01$. Den σ -Wert der Hammettschen Gleichung kann man als ein Mass für die Elektronenzugänglichkeit betrachten, die von einem Substituenten hervorgerufen wird. Auf Grund der geringen Grösse und relativen Lage der σ -Werte für *m*-B(OH)₂ und *p*-B(OH)₂ lässt sich voraussagen, dass die Dihydroxyborgruppe eine schwache *m*-dirigierende Wirkung haben sollte, die aber von den Wirkungen starker deaktivierender bzw. aktivierender Substituenten wie die Carboxygruppe und die Acetylaminogruppe völlig überlagert werden. Unsere experimentellen Befunde stehen damit im Einklang.

Die dirigierende Wirkung der Dihydroxyborgruppe wird in Essigsäureanhydrid vollständig verändert. Dies kann man folgendermassen erklären.

Das freie Dublett am Sauerstoff des Lösungsmittel wird in die Oktettlücke hineingezogen, wobei sich nach Gleichung I ein Dipol bildet.



Da die Achterschale des Bors aufgefüllt ist, kann es sich nicht mehr an mesomeren Elektronenverschiebungen beteiligen. Die negative Ladung des Bors erzeugt aber durch induktiven Effekt (+ I) eine höhere Elektronendichte im Kern.



Infolge mesomerer Elektronenverschiebungen werden die *ortho*- und *para*-Stellungen am stärksten beeinflusst und die Substitution wird deshalb an diesen Stellen erleichtert.

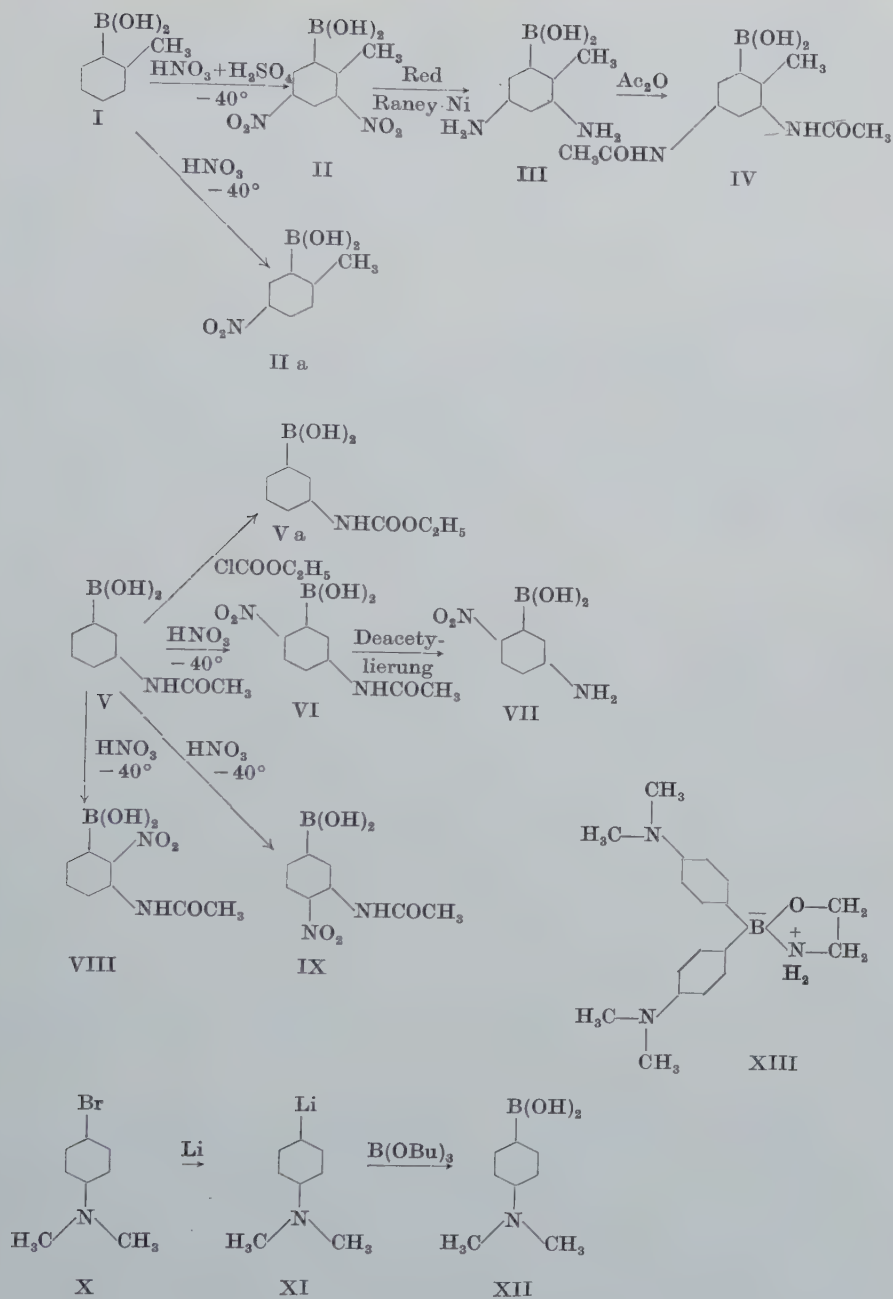
Nitrieren von Phenylborsäure in Essigsäureanhydrid ergab 70% *o*- und 10% *p*-Nitro-phenylborsäure. *m*-Acetylamino-phenylborsäure V lieferte bei der Nitrierung in Essigsäureanhydrid insgesamt 26% 2- und 4-Nitro-3-acetylamino-phenylborsäure. In rauchender Salpetersäure wurde za. 9% dieser Verbindungen erhalten.

Stabilität der B-C-Bindung

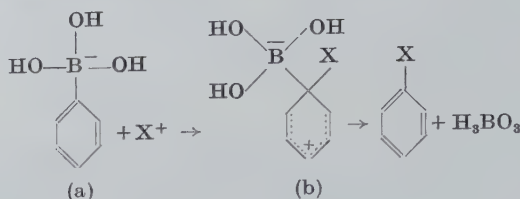
Es hat sich aus dem Verhalten der verschiedenen Arylborsäuren beim Nitrieren und bei anderen Umsetzungen herausgestellt, dass je positiver der Kern ist, d.h. je stärker die anderen Substituenten Elektronen heranziehen, desto widerstandsfähiger wird die B-C-Bindung gegen chemische Reagenzien. Die Monoamino-phenylborsäuren zerfallen von selbst an der Luft (2, 5). Die Acetylderivate sind stabil. Die Tolylborsäuren zersetzen sich sehr leicht beim Nitrieren, bloss vorsichtiges Behandeln führt zum erwünschten Nitroprodukt. Die Carboxy- und Nitro-derivate sind dagegen sehr stabile Verbindungen. 2-Nitro-4-carboxy-phenylborsäure wurde z. B. nach 5 Min. Erwärmen in rauchender Salpetersäure bei 80° unverändert zurückgewonnen. Sublimat spaltet gewöhnlicherweise in glatter Reaktion die Dihydroxyborgruppe unter Bildung von Arylquecksilberchlorid und Borsäure ab. 2-Nitro-4-carboxy-phenylborsäure wurde nach 5 Min. Kochen dagegen nicht angegriffen.

Beim Behandeln mit Salpetersäure wird die Dihydroxyborgruppe gegen eine Nitrogruppe ausgetauscht. Brom spaltet die Arylborsäure unter Bildung von Arylbromid und Borsäure. Die B-C-Bindung ist besonders gegen Silberionen empfindlich. Es ist denkbar, dass intermediär eine silberorganische Verbindung entsteht, die aber sofort von Wasser hydrolysiert wird, wobei Silber durch Wasserstoff ersetzt wird.

Aus diesen Tatsachen ist der Schluss gezogen worden, dass die Dihydroxyborgruppe durch elektrophile Substitution abgespalten wird, was natürlich um so leichter geht, je grösser die negative Ladung des bortragenden Kohlenstoffatoms ist. Amino- und Methylgruppen erhöhen seine negative Ladung, Nitro- und Carboxygruppen setzen sie herab.



Die Reaktionen lassen sich nach demselben Schema formulieren:



$\text{X}^+ = \text{NO}_2^+, \text{HgCl}^+, \text{Br}^+, \text{Ag}^+, \text{H}^+, \text{OH}^+.$

Unsere experimentellen Befunde stimmen gut mit den Ergebnissen von Kuivila und Mitarb. (7) überein, die die Bromolysegeschwindigkeit verschiedener Arylborsäuren gemessen haben. Die Prüfung der Hammettschen Gleichung ergab eine Gerade, was die Annahme weiter bestärkt, dass die geschwindigkeitsbestimmende Stufe die Bildung des Zwischenprodukts (b) ist. Wahrscheinlich beteiligt sich das Anion (a) an dieser Stufe, denn die Bromolyse verläuft schneller bei höherem pH.

Experimenteller Teil

Nitrierung von o-Tolylborsäure. a. 2-Methyl-3,5-dinitro-phenylborsäure II. Zu einer auf -40 bis -45° gekühlten Mischung von 12 ml rauchender Salpetersäure (Dichte 1,50, hellgelb) und 18 ml konz. Schwefelsäure wurden unter kräftigem Rühren 5 g gepulverte o-Tolylborsäure I in kleinen Portionen zugegeben. Bei dieser Temperatur bildete die Nitriersäure einen Brei, der während des Zusatzes geknetet werden musste. Es wurde sorgfältig darauf geachtet, dass der Zusatz von o-Tolylborsäure mit grösster Vorsicht geschah, da sonst die Reaktion so heftig verlaufen konnte, dass die Borverbindung zersetzt wurde. Die Temperatur wurde langsam auf -15° erhöht. Nach einstündigem Umrühren bei dieser Temperatur wurde die Lösung auf 200 ml Eiswasser gegossen. Die Dinitroverbindung II fiel aus. Man erhielt blättrige Kristalle, die aus Wasser umkristallisiert wurden. Schmp. 142° unter Wasserabgabe. Erstarrt wieder und schmilzt bei $205-210^\circ$. Ausbeute: 6,4 g oder 75%. Gef. C 37,3; H 3,19; N 12,20; ber. für $\text{C}_7\text{H}_7\text{O}_6\text{N}_2\text{B}$ (226,0) C 37,2; H 3,13; N 12,29.

Konstitutionsbeweis. 200 mg Nitroverbindung II wurden 10 Min. mit 6 ml 0,1 N Silbernitrat gekocht. Beim Erkalten fielen weisse Nadeln aus (Schmp. $69-70^\circ$). Sie wurden als 2,4-Dinitrotoluol identifiziert. Ein Mischschmelzpunkt ergab keine Depression. Ausbeute: 140 mg oder 87%.

b. 2 g gepulverte o-Tolylborsäure I wurden portionsweise in 8 ml Salpetersäure (Dichte 1,49, hellgelb) bei -40 bis -45° nitriert. Die Lösung wurde sofort rotbraun, was auf eine teilweise Zersetzung hindeutete. Nach dem Zusatz liess man die Lösung noch 15 Min. unter Umrühren bei derselben Temperatur stehen. Sie wurde auf Eiswasser gegossen, wobei sich eine schmierige Masse abschied (A). Nach Filtrieren wurde sie mit 8 N Natronlauge unter Kühlung neutralisiert. Ein teigiger Niederschlag fiel aus (B). (A) bestand teils aus etwas nicht umgesetztem Produkt, teils aus Nitrotoluolen. Es konnte auch eine geringe Menge einer Nitro-tolylborsäure nachgewiesen, aber nicht in reinem Zustand erhalten werden.

(B) ist in verdünnter Natronlauge löslich. Es gelang uns aber nicht, (B) durch Umkristallisieren weiter zu reinigen. Eine katalytische Hydrierung des getrockneten

Rohproduktes zeigte, dass es sich um ein Mononitroderivat handelte. Die dabei entstandene Amino-tolylborsäure war aber unbeständig und spaltete Borsäure ab. Aus dem Reaktionsgut konnte 0,4 g *p*-Acetylaminotoluol isoliert werden. Die Konstitution der Nitroverbindung ist deshalb 2-Methyl-5-nitro-phenylborsäure, IIa. Es wurde auch versucht, *o*-Tolylborsäure in Essigsäureanhydrid zu nitrieren. Es wurden aber nur schmierige Produkte erhalten.

Nitrierung von *m*-Tolylborsäure, 2-Thienylborsäure und α -Naphtylborsäure in rauchender Salpetersäure bei -40° oder in Essigsäureanhydrid ergaben nur schmierige Zersetzungsprodukte. Bei Nitrierung von *p*-Methoxyphenylborsäure wurde 2,4-Dinitroanisol in 71%iger Ausbeute isoliert.

2-Methyl-3,5-diamino-phenylborsäure III wurde in 81%iger Ausbeute von der entsprechenden Dinitroverbindung II durch katalytische Hydrierung in alkoholischer Lösung mit Raney-Nickel gewonnen. Gelbe, körnige Kristalle, die sich unter Aufschäumen bei 163° zersetzen, wurden erhalten. Leicht löslich in Alkohol. Gef. N 16,80; ber. für $C_7H_{11}O_2N_2B$ (166,0) N 16,88.

2-Methyl-3,5-*N,N'*-diacetylamino-phenylborsäure IV. Zu einer warmen Lösung von 0,2 g 2-Methyl-3,5-diamino-phenylborsäure III in 4 ml Eisessig wurden 1 ml Essigsäureanhydrid unter Schütteln zugegeben. Es trat sofort ein weisser Niederschlag auf, der beim Zusatz von Wasser in Lösung ging. Das Lösungsmittel wurde abdestilliert und der Rückstand aus Wasser umkristallisiert. Zersp. $> 340^{\circ}$. Leicht löslich in heissem Wasser. Gef. N 11,57; ber. für $C_{11}H_{15}O_4N_2B$ (250,1) N 11,21.

m-Acetylamino-phenylborsäure V. 26,5 g *m*-Nitro-phenylborsäure in 150 ml Äthanol wurden bei Zimmertemperatur und Atmosphärendruck katalytisch mit Raney-Nickel hydriert. Die Reduktion nahm 12 Stunden in Anspruch. 98% der theoretischen Wasserstoffmenge wurden absorbiert. Die alkoholische Lösung wurde vom Katalysator befreit, mit 50 ml Wasser versetzt und im Vakuum unter Stickstoff eingedampft. 100 ml Essigsäureanhydrid wurden zum öligen Rückstand zugegeben, wobei eine mässige Wärmeentwicklung eintrat. Die Lösung wurde 15 Min. auf 80° erwärmt und dann im Vakuum eingengt. Die Acetylaminoverbindung fiel als Brei aus. Sie wurde aus wenig Wasser mit Zusatz von aktiver Kohle umkristallisiert. Ausbeute: 19,5 g oder 69%. Der Schmelzpunkt war nach einmaligem Umkristallisieren etwa 250° . Die Reinheit war für die weiteren Umsetzungen genügend.

Nitrierung von *m*-Acetylamino-phenylborsäure V in rauchender Salpetersäure. 6-Nitro-3-acetylamino-phenylborsäure VI. Zu 15 ml rauchender Salpetersäure (Dichte 1,50, hellgelb) wurden bei -40 bis -45° 3 g feingepulverte *m*-Acetylamino-phenylborsäure V portionsweise zugegeben. Nach dem Zusatz liess man die Lösung 15 Min. unter Umrühren bei derselben Temperatur stehen. Sie wurde auf 200 ml Eiswasser gegossen, wobei sich sofort ein gelber Niederschlag abschied, der abfiltriert und im Exsiccator getrocknet wurde. Ausbeute: 3,5 g Rohprodukt. Es wurde drei Mal mit je 25 ml siedendem Xylol etwa drei Min. extrahiert. Man musste die heisse Xylollösung schnell filtrieren. Aus dem Filtrat fiel ein gelber Niederschlag aus (B), 0,35 g. Die auf dem Filter zurückgebliebene Substanz (A) wurde aus Wasser umkristallisiert. Gelbe Nadeln vom Zersp. $193-196^{\circ}$. Aus Acetonitril umkristallisiert, sintern sie bei etwa 200° , und zersetzen sich bei etwa 260° . Wenn sie wieder aus Wasser umkristallisiert werden, schmelzen sie bei $193-196^{\circ}$. Ausbeute: 2,6 g oder 69%. Die Substanz hat die Konstitution 6-Nitro-3-acetylamino-phenylborsäure, VI. Beim Abspalten der Dioxyborgruppe mit verdünnter Silbernitratlösung konnte in 76%iger Ausbeute *p*-Nitroacetanilid isoliert werden. Schmp. und Mischschmp. $211-212^{\circ}$. Gef. N 12,48; ber. für $C_8H_9O_5N_2B$ (224,0) N 12,50.

(B) wurde aus Wasser umkristallisiert, wobei zuerst gelbe blätterförmige Kristalle ausfielen (C). Nach längerem Stehen kamen kleine Mengen einer anderen Substanz (gelbe Nadeln) heraus (D). Die Ausbeute davon war allerdings sehr gering. (D) entsteht in grösserer Menge beim Nitrieren der Acetylaminoverbindung in Essigsäureanhydrid (siehe unten). In sowohl (C) als (D) sitzt die Nitrogruppe in *ortho*-Stellung zu der Aminogruppe. (C) sintert bei 140–150° und zersetzt sich bei etwa 195°. Es lässt sich auch aus Acetonitril umkristallisieren. Gef. N 11,47; ber. für $C_8H_9O_5N_2B \cdot H_2O$ (242,0) N 11,57.

Nitrierung von m-Acetyl-amino-phenylborsäure V in Essigsäureanhydrid mittels rauchender Salpetersäure. Zu einer Mischung von 15 ml Essigsäureanhydrid und 0,7 ml rauchender Salpetersäure (Dichte 1,50, hellgelb) wurden portionsweise bei –15° unter Umrühren 2 g *m*-Acetyl-amino-phenylborsäure zugesetzt. Die Temperatur wurde langsam auf 0° erhöht. Nachdem sich alles gelöst hatte, liess man noch 15 Min. unter Umrühren stehen. Das Reaktionsgut wurde auf 70 ml Eiswasser gegossen. Der Niederschlag wurde abfiltriert, getrocknet und vier Mal mit je 30 ml siedendem Xylol extrahiert. Der Rückstand (A) wurde einmal aus Wasser umkristallisiert und einmal aus Acetonitril. Er war mit (A) aus der obigen Nitrierung identisch. 0,3 g *6-Nitro-3-acetyl-amino-phenylborsäure* VI wurden isoliert.

Der Niederschlag aus dem Xylolfiltrat wurde in 11 ml heissem Äthylacetat gelöst. Bei langsamem Kühlen auf Zimmertemperatur fielen einheitliche gelbe körnige Kristalle aus, die noch einmal aus Wasser umkristallisiert wurden. Gelbe Blätter, die bei 140–150° sintern und sich bei etwa 195° zersetzen. Sie waren mit (C) identisch. Ausbeute: 0,4 g.

Die Äthylacetatlösung wurde zur Trockne eingengt und der gelbe Rückstand zweimal aus Wasser umkristallisiert. Lange gelbe Nadeln vom Zersp. 220–230°, je nach der Geschwindigkeit des Erhitzens. Sie sind mit (D) identisch. Ausbeute: 0,3 g. Gef. C 39,70; H 4,06; N 11,75; ber. für $C_8H_9O_5N_2B \cdot H_2O$ (242,0) C 39,76; H 4,58; N 11,57.

In (C) und (D) sitzt die Nitrogruppe in *ortho*-Stellung zu der Aminogruppe, denn beim Abspalten der Dihydroxyborgruppe entsteht in beiden Fällen *o*-Nitroacetanilid. Schmp. und Mischschmp. 91–92°. Die Verbindungen haben also folgende Konstitution: *2-Nitro-3-acetyl-amino-phenylborsäure* VIII und *3-Acetyl-amino-4-nitro-phenylborsäure* IX. Es wurde nicht weiter untersucht, ob (C) oder (D) die Konstitution VIII oder IX besaßen.

6-Nitro-3-amino-phenylborsäure VII. 0,25 g *6-Nitro-3-acetyl-amino-phenylborsäure* VI wurden mit 3 ml 2 N Salzsäure 7 Min. gekocht. Die Lösung wurde mit 6 N Natronlauge auf pH 4–6 neutralisiert. Gelbe Nadeln fielen aus. Ausbeute: 0,12 g. Aus Wasser umkristallisiert zersetzt sich das Amin bei 250–255° unter Verkohlungs, fängt aber schon bei etwa 150° an, dunkel zu werden. Es löst sich in heissem Nitromethan, fällt aber nach kurzer Zeit als rote Kristalle aus der warmen Lösung wieder aus. Beim Stehen in der Kälte bekommen die Kristalle langsam ihre gelbe Farbe zurück. Gef. C 37,71; H 4,32; N 14,52; ber. für $C_6H_7O_4N_2B \cdot 1/2 H_2O$ (191,0) C 37,73; H 4,22; N 14,67.

Die drei *Mono-nitro-phenylborsäuren* wurden nach der Methode von Seaman und Johnson (2) dargestellt. Aus 10 g Phenylborsäure wurden nach der Essigsäureanhydridmethode 8,5 g *o*-Nitro-phenylborsäure und 1,1 g *p*-Nitro-phenylborsäure erhalten.

m-Carbäthoxyamino-phenylborsäure Va. 0,30 g *m*-Acetyl-amino-phenylborsäure V wurden durch 10 Min. langes Kochen in 5 ml 2 N Salzsäure deacetyliert. Die Lösung

wurde mit Soda neutralisiert. Es wurden noch 0,3 g wasserfreie Soda und, unter Kühlung, 0,25 g Chlorameisensäureäthylester zugesetzt. Nach kräftigem Schütteln wurde schwach angesäuert. Der weisse blättrige Niederschlag wurde aus Wasser umkristallisiert. Schmp. 202–203°. Gef. N 6,53; ber. für $C_9H_{12}O_4NB$ (209,0) N 6,70.

p-Dimethylamino-phenylborsäure XII. *p*-Lithium-dimethylanilin, aus 10 g *p*-Bromdimethylanilin und 0,9 g Lithiumspänen in 100 ml Äther dargestellt, wurde unter Stickstoff tropfenweise zu einer auf -70° gekühlten und gut umgerührten Lösung von 11,5 g *n*-Tributylborat in 25 ml trockenem Äther gegeben. Die Temperatur durfte nicht über -65° steigen. Man liess die Lösung über Nacht stehen und langsam Zimmertemperatur erreichen. 25 ml zerstoßenes Eis wurden zugegeben, worauf unter Schütteln 20 ml 6 *N* Salzsäure zugefügt wurden. Die ätherische Schicht wurde von der wässrigen abgetrennt und mit 10 ml Wasser gewaschen. Die vereinigten blaugefärbten Wasserlösungen wurden unter Kühlung mit 5 *N* Natronlauge auf pH 4–6 neutralisiert (eventueller Niederschlag wurde abfiltriert) und mit 2 ml Aminoäthanol in 5 ml Wasser portionsweise versetzt, um die Diarylborsäure auszufällen (8). Es bildete sich ein dicker, hellblaugefärbter Niederschlag, der abfiltriert und getrocknet wurde. Ausbeute: 6,0 g. Rohschmp. 130–145°. Das Filtrat wurde auf ein kleines Volumen eingengt, daraus konnte aber keine bororganische Verbindung mehr isoliert werden. Die ätherische Schicht enthielt nur etwas Borsäure. Sämtliche Operationen wurden unter Stickstoff durchgeführt. Der Niederschlag, der mit grösster Wahrscheinlichkeit aus Bis-*p*-dimethylamino-phenylborsäure-aminoäthylester XIII bestand, wurde aus Äthanol-Wasser (3:2) unter grossem Substanzverlust umkristallisiert. Zersp. 240–260°. Wahrscheinlich zersetzt sich der Ester unter Bildung von *p*-Dimethylamino-phenylborsäure, Dimethylanilin und Aminoäthanol. Nach Umkristallisieren aus Benzol schmolz die Substanz XII bei 270–275°. Gef. C 65,60; H 6,81; B 7,16; ber. für $C_8H_{10}ONB$ (anhydriisch, 147,0) C 65,36; H 6,86; B 7,36. Sie zersetzt sich sehr langsam an der Luft unter Bildung von Borsäure und Dimethylanilin. Ausbeute: 1,1 g.

Zusammenfassung

Es wird das Nitrieren einiger Arylborsäuren beschrieben. Die dirigierende Wirkung der Dihydroxyborgruppe und die Stabilität der Bor-Kohlenstoff-Bindung werden näher besprochen.

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LITERATUR

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Tryckt den 25 januari 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

Sectioned electrophoresis column for crude preparative fractionations of large quantities

By JERKER PORATH

With 3 figures in the text

When dealing with highly viscous solutions, the elution of zones over great distances in a powder-stabilized electrophoresis column will cause very large spreading, which may spoil an experiment. The author has encountered this kind of complication in attempts to fractionate crude extracts of parathyroid glands. Such solutions, however, can be satisfactorily applied in the middle of a *sectioned* column.

During the past few years preparative electrophoresis in vertical columns has been intensively studied at this Institute (1-5). Very effective separations of mixtures of charged substances of high and low molecular weight can now be obtained. However, it has been shown to be convenient—for practical reasons—to perform a preliminary separation of the natural raw material into a few fractions so as to enable a more effective utilization of the principles of electrophoretic separation by allowing a unidirectional migration of the material. This type of crude fractionation can often be obtained with ion exchange columns, but may also be performed electrophoretically when reasonable quantities are involved (10-100 g).

The apparatus described here has been designed in order to meet these demands. To a certain extent it resembles the apparatus described by Butler and Stephen (6) with respect to assembly, but it deviates considerably with respect to the manual operations.

Experimental

Apparatus

Principle.—The electrophoresis is performed in a horizontal column consisting of three or more identical sections (AI, AII, etc., see Fig. 1). The sections are connected via the side arms (B) and (C) with the electrode vessels (E1) and (E2). The column is cooled by running water in the trough (D).

Column Sections (Figs. 1 and 2).—These are constructed of 35-40 cm long pyrex tubes of 3 cm diameter. One end is terminated at (A1) a female joint (standard, ground joint no. 34, as are the remaining joints), the inner surface of which has a ground groove (A2). (This groove can be omitted on the first section, AI.) At an aperture in the anterior (upper) end of the groove, approximately at the middle of the joint, there is a side arm (A3) with a stop-cock (A4). The other end of the section is terminated by a male joint (A5), provided with several inwardly directed glass rods for sup-

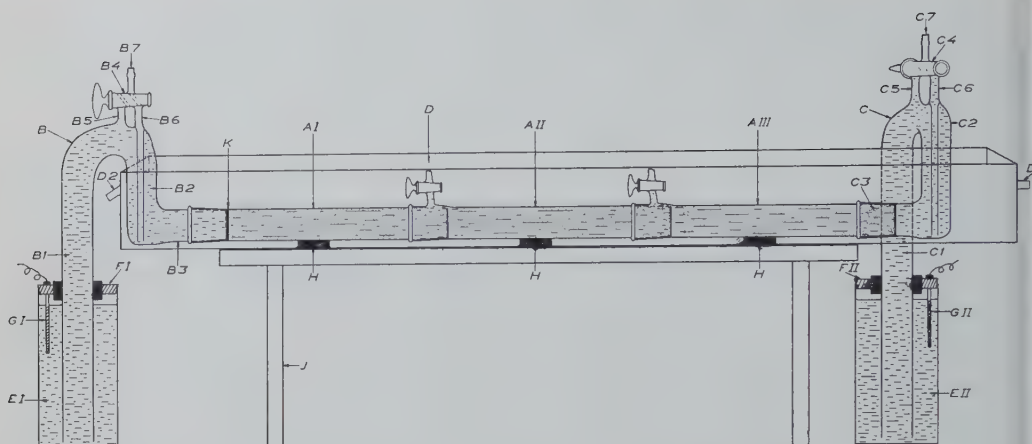


Fig. 1. Schematic drawing of the electrophoresis apparatus assembly.

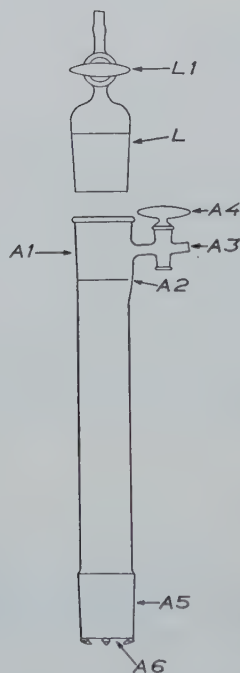


Fig. 2. Column section (A) and a head (L) used during elution.

porting a thick filter paper disc (A6) which in turn supports the buffer and the medium in the column.

The connecting arms.—The side arm (B) consists of a U-tube with long and short vertical arms (B1 and B2). At (B2) a short horizontal arm is connected via (B3) to a female joint at its open end. A two-way stop-cock connected with two narrow

tubes (B5 and B6) is located at the upper end of the U-tube. (B6) extends vertically through (B2) with its lower opening one mm from the glass wall in a slight hollow in the corner between (B2) and (B3). The connecting arm (C) is analogously constructed, but (C3) is normally directed through the plane through (C1) and (C2) and is terminated by a femal joint.

The electrode vessels.—These consist of two cylinders (EI) and EII) with loose-fitting covers (FI and FII) through wich protrude platinum threads wound around glass rods (GI and GII).

Cooling trough.—The electrophoresis tube is immersed in a 1.5 meter long trough of perspex or metal (D), filled with cold running water flowing through the inlet and outlet tubes (D1) and (D2). The supports (H) lift the electrophoresis tube from the bottom of the trough. (D) rests on a wooden bench (J).

Current source.—Stabilized direct current from a rectifier.

Operation

Packing, application of the substance, electrophoresis and elution are performed in accordance with the factors discussed earlier (Flodin and Porath (2), Porath (3), Flodin and Kupke (4), Porath (5)). Each section is packed with the supporting medium up to the lower edge of the female joint. A suitable solution of the substance is introduced into one of the sections. The space between the packing and the upper end of the groove (A2) is filled with buffer solution. The sections are connected in vertical position so that the excess buffer flows out through the openings at (A3) after which the stopcocks (A4) are closed. A 1–2 mm thick filter paper disc (K) is laid on the surface of the supporting medium of the upper section. The column is lowered to the horizontal position and connected to the electrode vessels (B) and (C). The empty trough (D) is placed under the column and allowed to rest on the supports. The electrolyte is aspirated from the cylinder into (B) by suction at (B7) and (C) is filled in a corresponding manner. The trough is filled with cooling water, and the current is applied.

After electrophoresis (B4) and (C4) are opened. The trough is lowered and set aside. The buffer solution in (B2) and (B3) is removed via (B6) by suction at (B7). (C2) and (C3) are emptied in an analogous manner.

The stop-cocks (A4) are opened and the sections separated and placed in a vertical position. Each section is provided with an upper part (L) narrowing upward to a tube with a stop-cock (L1). (L) is connected with a buffer reservoir and the buffer with the fractionated substances is displaced from the column with fresh buffer, at the same time regenerating the sections.

Separation of an autolyzed yeast extract into neutral, acid and basic fractions

The following buffer systems were used:

Buffer *a*: 0.1 molar acetic acid and 0.3 molar pyridine

Buffer *b*: 1 molar and 3 molar pyridine.

Buffers *a* and *b* have practically the same pH (5.8) and a conductivity of 3.47×10^{-3} and $12.6 \times 10^{-3} \text{ ohm}^{-1} \text{ cm}^{-1}$ at 0.5°C , respectively. Ten grams of dehydrated yeast extract (Difco Bacto Yeast Extract) was dissolved in 100 ml of buffer *a*. This solution, the conductivity of which was $8.78 \times 10^{-3} \text{ ohm}^{-1} \text{ cm}^{-1}$ at 0.5°C , was intro-

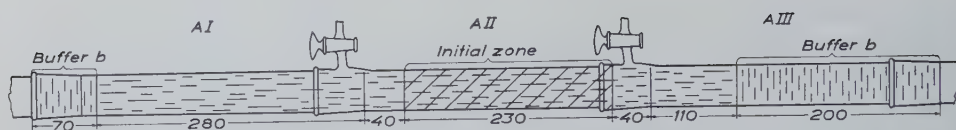


Fig. 3a. Approximate positions of the initial zone and the buffer boundaries at the start of electrophoretic separation of 10 g of yeast extract as described in the text. Distance in mm.

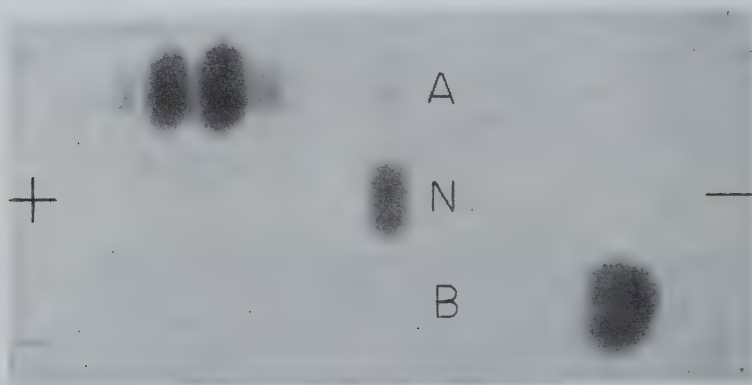


Fig. 3b. Paper electropherogram of the acidic (A), natural (N) and basic (B) fractions obtained from the experiment referred to in Fig. 3a.

duced into the middle section (see Fig. 3a) surrounded by buffer *a*. Buffer *b* in the side sections functioned as a barrier electrolyte. The approximate positions of the zone and layer borders are indicated in Fig. 3a. The electrode vessels and side arms were filled with buffer *b* and 600 volts (*ca.* 250 mA) were applied between the electrodes. After 18 hours electrophoresis the column was dismantled and each section was eluted separately. The solutions were dried *in vacuo* over conc. H_2SO_4 and NaOH pellets to constant weight. Section AI contained the acidic fraction, A (3.85 g), section AII the neutral, N (1.55 g), and AIII the basic fraction, B (4.85 g). Thus, all of the material applied to the column could be accounted for. Samples of the fractions were tested by paper electrophoresis in buffer *a*, with 15 cm wide, Whatman 3 MM paper in an apparatus similar to that of LKB. After 15 hours of electrophoresis (5 mA), the paper was dried, dipped into a 0.1% solution of ninhydrin in acetone and subsequently warmed. Fig. 3b shows the developed ninhydrin spots.

Discussion

A crude separation of charged material of low molecular weight, such as a mixture of amino acids, can be performed by means of electrodialysis in a multi-chambered apparatus, for example, according to Pauli (7), Theorell and Åkesson (8), or Synge (9). A difficult problem in electrodialysis in apparatus of such types is the regulation of the electro-osmosis. In natural extracts containing substances of different molecular weight—from amino acids to proteins, polynucleotides, etc.—use of the electrodialysis apparatus becomes complicated by the fact that substances of high molecular

weight do not pass through the membranes and substances of intermediary size fasten in the membranes usually employed, gradually filling up the pores.

The method described here does not have these limitations, providing that a suitable porous material is chosen. In cellulose powder the electro-osmosis is so slight in all of the ordinary buffer systems (except possibly at a high pH combined with an extremely low ionic strength), that it does not have to be considered. The fractionation of substances of both high and low molecular weight can be predicted from electrophoresis in free solution. As a rule, adsorption on the cellulose powder occurs only to a limited extent and can be neglected in preparative amino acid, peptide, and protein fractionation.

Compared with the columns previously described by the author, the construction of the present apparatus is simpler and the voltage-loss is less. Naturally this is at the expense of a reduced separation efficiency. An inexpensive rectifier can thus be used and the construction of the apparatus for fractionation of large quantities is cheap. The manual operations present no difficulties. With wider columns it should be possible to increase the capacity 5–10 times, while retaining the same simple construction.

The separation described above, of ninhydrin-positive material in an autolysed yeast extract, demonstrates that a satisfactory group separation can be obtained in spite of the unavoidable distortion of the electrical field and the less effective electrolyte stabilisation in the section joints. Experiments with dyes have shown that the zone spreading in a small number of junctions is of little significance as compared with the spreading along the lower side of the column caused by the convectational transport. The use of sectional vertical columns in some cases for particularly effective separations will be described elsewhere.

ACKNOWLEDGEMENTS

The author is indebted to Professor Arne Tiselius for valuable discussions and to Mr. Sture Jerstedt for able technical assistance. This work is part of a program on the development of zone electrophoresis methods at this Institute. It has been financially supported by grants from the Swedish Natural Science Research Council, the Rockefeller Foundation and the Wallenberg Foundation.

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The chemistry of arylboric acids. V*

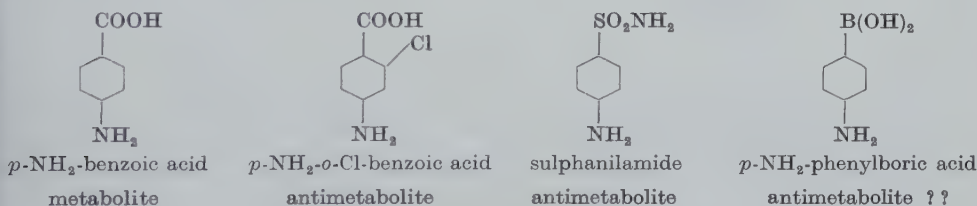
Effects of arylboric acids on micro-organisms and enzymes

By KURT TORSSELL

With 7 figures in the text

A. Effects of arylboric acids on bacteria

Parts I-IV of this series deal with the syntheses and properties of a number of arylboric acids. Some of them have structural resemblances to certain antagonists of the essential metabolite, *p*-aminobenzoic acid (PABA), e.g. sulphonamides and some derivatives of benzoic acid. It was therefore of interest to test them for their bacteriostatic and bacteriocidal action. The derivatives of phenylboric acid containing an amino group in the *para*-position were of special interest.



The structures of the substances are shown above. It seems possible that the acid dihydroxyboron group could replace the carboxyl group.

The simple *p*-amino-phenylboric acid is unfortunately unstable in air but the acetylated derivative is stable and has been tested, together with about fifty other compounds, for activity against *Escherichia coli* (Gram negative), *Staphylococcus aureus* (Gram positive) and *Mycobacterium phlei* (acid-fast). In order to avoid the competitive effect of PABA, a peptone-free culture medium was used and sulphathiazole and sulphadiazine were used as reference substances. The sulphathiazole index, i.e. the ratio concentration of drug/concentration of sulphathiazole, which just results in bacteriostasis, was calculated. As the growth of bacteria was not tested in concentrations of arylboric acid higher than 1:3000, the index may be still larger for those substances not showing any inhibiting effect at this concentration.

It appears from Table 1 that the simple phenylboric acid [no. 19] is more active than *p*-acetyl-amino-phenylboric acid [39], and as active as some of the *p*-amino-derivatives [5, 6, 12, 13]. The activity of compound 6 was also tested in the presence

* IV. *Arkiv Kemi* 10 (1957), 513.

Table 1. Effect of arylboric acids on bacteria.

In the structural formula the dihydroxyboron group, $-B(OH)_2$, is omitted. The free bond indicates its position. The figures in the concentration columns denote the dilution, at which only a very slight growth was detectable. 1 = 1:3000, 2 = 1:6000, 3 = 1:12,000, 4 = 1:24,000, 5 = 1:48,000, 6 = 1:96,000, 7 = 1:192,000, 8 = 1:384,000. The letters in the same columns signify growth on streak plates when inoculated with a completely inhibited culture. *a* = no growth; *b* = a few colonies; *c* = slight growth; *d* = rich growth. STA index denotes sulphathiazole index (see the text).

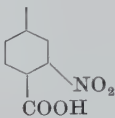
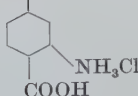
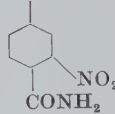
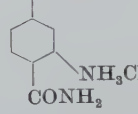
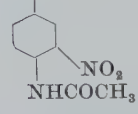
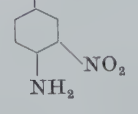
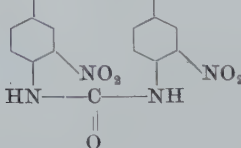
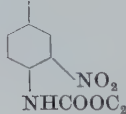
No.	Arylboric acid	<i>E. coli</i>		<i>S. aureus</i>		<i>M. phlei</i>	
		Conc.	STA index	Conc.	STA index	Conc.	STA index
1		<1	>16	1	128	4, <i>d</i>	8
2		<1	>16	<1	>128	<1	>64
3		<1	>16	2	64	4, <i>d</i>	8
4		1	16	1	128	1	64
5		1	16	4, <i>c</i>	16	4, <i>b</i>	8
6		2	8	3	32	4, <i>b</i>	8
7		1	16	4, <i>c</i>	16	2	32
8		1	16	<1	>128	<1	>64

Table 1 (continued).

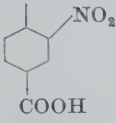
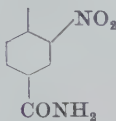
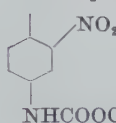
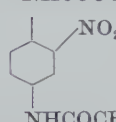
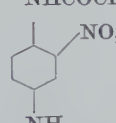
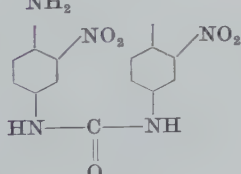
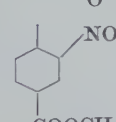
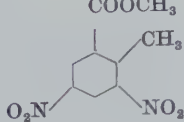
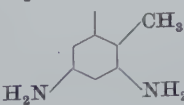
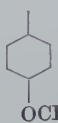
No.	Arylboric acid	<i>E. coli</i>		<i>S. aureus</i>		<i>M. phlei</i>	
		Conc.	STA index	Conc.	STA index	Conc.	STA index
9		<1	>16	<1	>128	1	64
10		<1	>16	2	64	2	32
11		<1	>16	2	64	4, c	8
12		<1	>16	2	64	2	32
13		<1	>16	<1	>128	<1	>64
14		<1	>16	2, b	64	4, b	16
15		4, c	2	2	64	4, b	16
16		<1	>16	2, c	64	3, b	16
17		<1	>16	<1	>128	<1	>64
18		1	16	1	128	1	64

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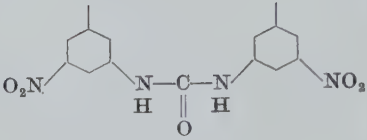
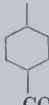
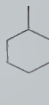
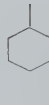
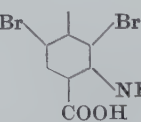
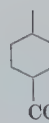
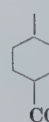
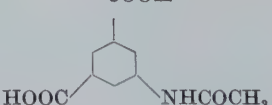
No.	Arylboric acid	<i>E. coli</i>		<i>S. aureus</i>		<i>M. phlei</i>	
		Conc.	STA index	Conc.	STA index	Conc.	STA index
19		< 1	> 16	3, c	32	4, d	8
20		1	16	2	64	2	32
21		< 1	> 16	1	> 128	< 1	> 64
22		< 1	> 16	1	128	1	64
23		< 1	> 16	1	> 128	< 1	> 64
24		< 1	> 16	1	128	1	64
25		1	16	1	128	2	32
26		< 1	> 16	< 1	> 128	< 1	> 64
27		< 1	> 16	4, d	16	4, d	8
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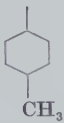
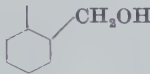
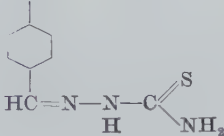
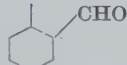
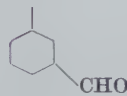
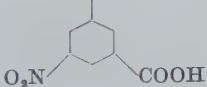
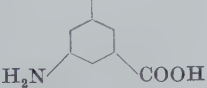
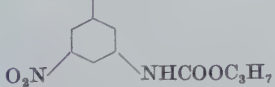
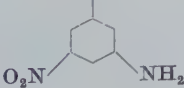
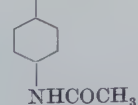
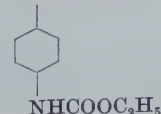
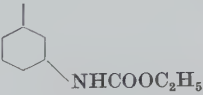
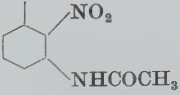
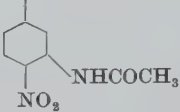
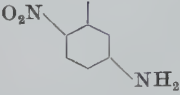
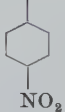
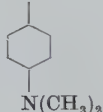
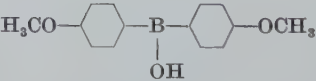
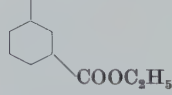
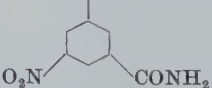
No.	Arylboric acid	<i>E. coli</i>		<i>S. aureus</i>		<i>M. phlei</i>	
		Conc.	STA index	Conc.	STA index	Conc.	STA index
30		< 1	> 16	1	128	2	32
31		5, d	1	5, b	8	7, b	1
32		1	16	2, c	64	2, b	32
33		4	2	4	16	4, a	8
34		1	16	3, c	32	5, c	4
35		< 1	> 16	< 1	> 128	< 1	> 64
36		< 1	> 16	< 1	> 128	< 1	> 64
37		< 1	> 16	3, b	32	5, c	4
38		1	16	5, c	16	5, d	4
39		< 1	> 16	1	128	2	32
40		< 1	> 16	2	64	1	64

Table 1 (*continued*).

No.	Arylboric acid	<i>E. coli</i>		<i>S. aureus</i>		<i>M. phlei</i>	
		Conc.	STA index	Conc.	STA index	Conc.	STA index
41		< 1	> 16	1	128	5, c	4
42 or 43		< 1	> 16	3, d	32	5, c	4
43 or 42		2, d	8	2, c	64	3, b	16
44		< 1	> 16	< 1	> 128	1	64
45		1	16	3, a	32	3, d	16
46		< 1	> 16	2, b	64	1	64
47		2	8	1	128	1	64
48		< 1	> 16	2	64	4, d	8
49		1	16	1	128	1	64
50	Sulphathiazole	5, d	1	8, b	1	7, b	1
51	Sulphadiazine	4, d	2	7, c	2	7, b	1

of 0.2×10^{-5} *M* PABA but no antagonistic effect could be detected. From this, and from the general lack of activity of the compounds, the conclusion may be drawn that they do not compete with PABA. The dihydroxyboron group is unable to replace the carboxyl group, which was also the case when the derivatives were tested for auxin activity (ref. 1). The derivatives are bacteriostatic in action, since inoculation of agar plates by the completely inhibited cultures gave rise to some growth, in some cases only a few colonies [nos. 5, 6, 14, 15, 16, 31, 32, 33, 37].

The most active substance found is *o*-hydroxymethyl-phenylboric acid which possesses an activity of about the same order as sulphathiazole. Its index varies between 1 and 8 for the bacteria tested. It may be mentioned here that this substance is also toxic to wheat seedlings at a concentration of about 10^{-5} *M* (1). The sensitivity of the bacteria decreases in the following order:

M. phlei > *S. aureus* > *E. coli*.

Most substances are inactive or very slightly active and are therefore without chemotherapeutic interest. The index varies between 128 and 32, i.e. sulphathiazole is about hundred times more active than most arylboric acids. Johnson *et al.* (2, 3) have tested some other arylboric acids without finding any remarkable activity. The toxicity of phenylboric acid has been estimated by Caujolle *et al.* (4), who found that the lethal dose, LD₅₀, was 560 mg/kg intraperitoneally in the mouse and 284 mg/kg in the guinea pig, i.e. phenylboric acid is rather harmless to animals. It can therefore be expected that the aromatic dihydroxyboron group will not be toxic to any noticeable extent in other derivatives.

From the chemotherapeutic point of view nitro derivatives are of little value because of their toxicity to the host. Most of the substituted phenylboric acids that have been synthesised are nitro derivatives but work is in progress on the preparation of some halogen-substituted derivatives. The introduction of a hydroxy group is also of interest in this connection.

B. Effects of arylboric acids on yeast, enzymes and sea urchin eggs

It has been stated that phenylboric acid forms complexes with sugars (5, 6, 7), and the micro-organisms or enzymes tested in this part were chosen with this in mind. Yeast contains some essential enzyme systems regulating the breakdown of sugars; invertase, α -amylase and phosphorylase are of importance in the carbohydrate metabolism.

The jelly coat of sea urchin eggs consists of 75–80 per cent of polysaccharides esterified with one sulphate group per monosaccharide residue (8). The experiments with this material were carried out to study the effect of possible complex formation between the polysaccharide and phenylboric acid on the fertilisation rate.

I. Effects of phenylboric acid on baker's and brewer's yeast

The effect of phenylboric acid (or its sugar complexes) upon the fermentation activity of fresh baker's yeast and dried brewer's yeast (bottom yeast) has been studied. 10^{-2} *M* boric acid is without effect on yeast, but this does not exclude the possibility that the introduction of the lipophilic phenyl group can change its behaviour. In fresh yeast the cells are intact, while most of them are destroyed in the dried yeast.

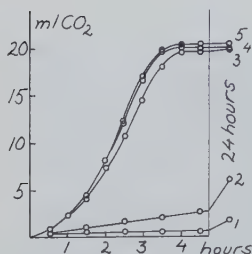


Fig. 1. Fermentation of maltose by fresh baker's yeast in the presence of phenylboric acid. Maltose (100 mg) + yeast suspension (1 ml), (10 g yeast in 30 ml water) + phenylboric acid. Final volume 2.5 ml. Curve 1, run with 5×10^{-2} M phenylboric acid; 2, with 2.5×10^{-2} M; 3, with 5×10^{-3} M; 4, with 5×10^{-5} M; 5, control, no phenylboric acid.

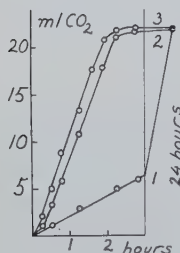


Fig. 2. Fermentation of glucose by fresh baker's yeast in the presence of phenylboric acid. Curve 1, run with 5×10^{-2} M phenylboric acid; 2, with 5×10^{-3} M; 3, control, no phenylboric acid. Every assay mixture contains 100 mg glucose.

No significant difference of action can be observed when fresh yeast or dried yeast is treated with phenylboric acid. Fig. 1 shows the action of phenylboric acid on the fermentation of maltose by fresh baker's yeast. At concentrations $> 10^{-2}$ M the fermentation is strongly inhibited (curves 1 and 2). This concentration is so high that the action can be referred to an undefined toxic effect of the substance. Small concentrations, $< 5 \times 10^{-3}$ M, have no effect (curves 3 and 4). Curve 5 is the control. Fig. 2 represents the fermentation of glucose. Even here it is inhibited only by high concentrations. It appears from curve 1 that the enzymes are not destroyed by the high phenylboric acid concentration (5×10^{-2} M), for after 24 hours all glucose has been fermented (pH 3.5–4.5). Fig. 3 shows the fermentation of glucose by dried brewer's yeast in a phosphate buffer, pH 5.8, at various concentrations of phenylboric acid. The fermentation is unaffected at low concentrations, $< 5 \times 10^{-3}$ M, and inhibited at higher ones. From these experiments it can be concluded that phenylboric acid at concentrations $\leq 10^{-3}$ does not affect the yeast enzymes regulating the alcohol fermentation. It may however be pointed out that the yeasts and carbohydrate enzymes have not been incubated for any length of time with phenylboric acid. The yeast or enzyme has been added to the substrate containing phenylboric acid. It is

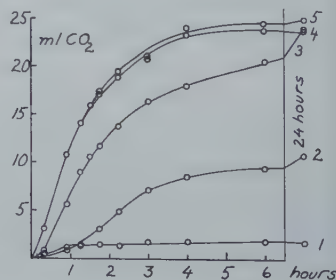


Fig. 3. Fermentation of glucose (100 mg) by dried brewer's yeast in the presence of phenylboric acid. Curve 1, control, autofermentation of yeast, no glucose; 2, run with 5×10^{-2} M phenylboric acid; 3, with 2×10^{-2} M; 4, with 5×10^{-4} M; 5, control, no phenylboric acid. The assay mixtures contain 150 mg dried yeast.

possible that the effect may be more marked if phenylboric acid is permitted to act upon the enzyme before it is mixed with its substrate.

Phenylboric acid has however another unexpected property. When fresh baker's yeast is treated with $5 \times 10^{-2} M$ phenylboric acid the autofermentation increases. 1 g fresh yeast liberates thereby in 24 hours 15.8 ml carbon dioxide, which corresponds to ~ 75 mg glucose, i.e. ~ 25 per cent of the dry weight. In the control 0.5 ml is evolved. The content of fermentable carbohydrates in yeast varies considerably but the high autofermentation indicates that phenylboric acid causes almost complete fermentation of the stored carbohydrates. The reasons for this have not yet been investigated.

The dry yeast (bottom yeast) is prepared according to Bamann and Myrbäck (9) and the course of fermentation is followed by volumetric measurement of the carbon dioxide evolved (9, p. 2150).

II. Effects of phenylboric acid on enzymes

(a) *Invertase. Experimental.*—The activity of the yeast invertase was determined according to Myrbäck and Willstaedt (10) with slight modifications. Saccharose (2.60 g) dissolved in water, 1 *M* acetate buffer (10 ml), pH 4.5, and suitable amounts of 0.1 *M* phenylboric acid or boric acid solution were made up to 100 ml and equilibrated at 30°. Enzyme solution (1 ml) was added and the mixture shaken well. At suitable intervals 10 ml samples were pipetted into 1.00 *M* sodium hydroxide (5 ml). This solution was neutralised again after some minutes with 1.00 *M* hydrochloric acid (5 ml). The optical rotation was measured in a 2 dm tube (no mutarotation).

Fig. 4 shows that the invertase activity is almost unaffected by $5 \times 10^{-2} M$ phenylboric acid, curve 3, and boric acid, curve 2. Curve 1 is the control.

It is necessary to neutralise the alkaline sugar solutions after destroying the enzyme with alkali because at high pH complex formation between saccharose and the boric acids influences the optical rotation so that comparison with the control is impossible. The broken lines indicate theoretical values for the rotation before and after inversion.

(b) *α -amylase.*—The activity of purified α -amylase from malt was determined by viscosimetric measurements of the breakdown of starch at various concentrations of phenylboric acid.

A homogeneous autoclaved potato starch solution was prepared. Acetate buffer (1 ml, 1 *M*, pH 5.2), starch solution (10 ml) and phenylboric acid solution (5 ml) or in the control water, were mixed and equilibrated at 30°. Enzyme solution (1 ml) was

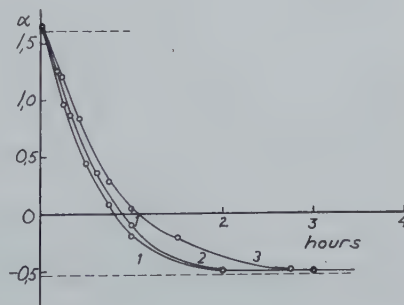


Fig. 4. Effect of phenylboric acid, $5 \times 10^{-2} M$ (curve 3) and boric acid, $5 \times 10^{-2} M$ (curve 2) on the inversion rate of saccharose by yeast invertase. Curve 1 is the control.

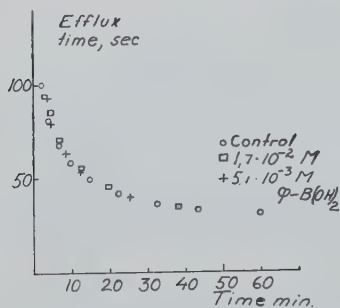


Fig. 5. Effect of phenylboric acid on the activity of α -amylase.

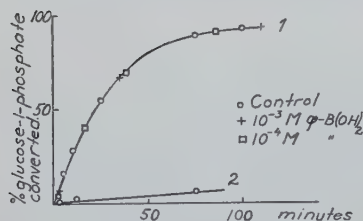


Fig. 6. Effect of phenylboric acid on the rate of glucose-1-phosphate conversion by potato phosphorylase.

added and the mixture shaken rapidly. 5 ml of the solution was pipetted into an equilibrated viscosimeter and the efflux time measured at intervals.

Fig. 5 shows the efflux time (extent of degradation) as a function of time. Addition of phenylboric acid did not change the activity of α -amylase.

Contrary to our results Gayrel (16) found that amylase from malt was inhibited by M/61 phenylboric acid to an extent of 13 %, calculated on basis of colourimetric estimations, and to 38 %, calculated on basis of determinations of reducing sugars formed.

(c). *Phosphorylase*.—Potato phosphorylase was prepared according to Sumner and Myrbäck (11). The orthophosphate liberated from Glucose-1-phosphate during the amylose synthesis (12) was measured by the procedure of Teorell (13). It can be seen from Fig. 6 that the addition of 10^{-4} – 10^{-3} M phenylboric acid does not affect the activity of the enzyme or the equilibrium between glucose-1-phosphate and starch, curve 1. To ensure that phosphatase was not responsible for the phosphate liberated, the primer (Lintner starch) was omitted in one experiment. The amount of phosphoric acid found was, as expected, very small (curve 2).

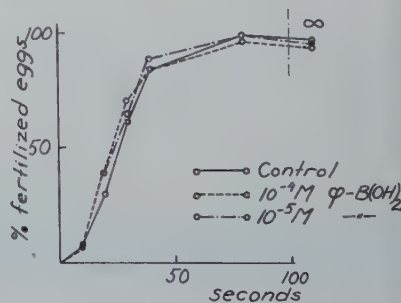


Fig. 7. Effect of phenylboric acid on the fertilisation rate of sea urchin eggs.

Table 2. The effect of some arylboric acids on the activity of cholinesterase prepared from human blood serum.

Compound	I_{50} = molar concentration giving 50 % enzyme inhibition
Phenylboric acid	4×10^{-6}
<i>p</i> -Cl-phenylboric acid	1.4×10^{-5}
<i>p</i> -NO ₂ -phenylboric acid	3×10^{-5}
<i>o</i> -Hydroxymethyl-phenylboric acid	5×10^{-5}
<i>p</i> -Methoxy-phenylboric acid	5×10^{-4}
<i>p</i> -Acetyl-amino-phenylboric acid	$> 10^{-3}$
<i>isobutyl</i> boric acid	$> 10^{-3}$
Physostigmine	3×10^{-8}
D F P	1×10^{-8}

(d). *Cholinesterase*.—The cholinesterase used was obtained from human blood serum and its activity was determined according to Augustinsson (14). It was incubated with the substance to be tested for fifty minutes before the addition of the substrate (acetylcholine chloride). The concentration of inhibitor given in Table 2 is that present in the incubation mixture. It can be seen from Table 2 that phenylboric acid possesses a rather high inhibitory activity but its derivatives, whether substituted with electron-repelling or with electron-attracting substituents, are less active. The I_{50} values signify the molar concentration of the compound giving 50 per cent enzyme inhibition. Phenylboric acid is about hundred times less active than the reference substance physostigmine.

III. Effects of phenylboric acid on the fertilisation rate of sea urchin eggs

The fertilisation experiments were carried out according to Hagström and Hagström (15). It appears from Fig. 7 that the addition of phenylboric acid has no significant effect upon the fertilisation rate. Naphthylboric acid likewise shows no activity.

Summary

1. *Bacteria*.—Most of the arylboric acids tested are inactive or very slightly active against bacteria *in vitro* at a dilution of 1:3000. They are bacteriostatic in action and it appears from their behaviour that they do not compete with PABA. The sensitivity of the bacteria to arylboric acids decreases in the following order: *M. phlei* > *S. aureus* > *E. coli*.

The most active derivative found was *o*-hydroxymethyl-phenylboric acid which inhibits the growth of bacteria only slightly less than sulphathiazole or sulphadiazine.

2. *Yeast*.—The effect of phenylboric acid on the fermentation of glucose and maltose by fresh baker's and dried brewer's yeast (bottom yeast) has been studied. High concentrations of the agent, $> 5 \times 10^{-3}$ *M*, inhibit the fermentation activity, while low concentrations are without effect. No significant difference in reaction of the

two yeast types could be detected when they were treated with phenylboric acid. However, the autofermentation of fresh baker's yeast in 5×10^{-2} *M* phenylboric acid was strongly promoted.

3. *Enzymes*.—(a) The activity of yeast *invertase*, α -*amylase* from malt, and potato *phosphorylase* is unaffected by phenylboric acid in concentrations $\leq 5 \times 10^{-3}$ *M*, i.e. its sugar complexes are also without effect on the enzyme activity.

(b) *Cholinesterase* is inhibited by some arylboric acids. Phenylboric acid is the most active and has an I_{50} value of 4×10^{-6} *M*. The enzyme was prepared from human blood serum.

4. *The fertilisation rate of sea urchin eggs*. This is unaffected by phenyl- and naphthylboric acid at concentrations $\leq 10^{-4}$ *M*.

I wish to express my gratitude to the head of the institute, Prof. Karl Myrbäck, for valuable discussions and suggestions concerning the enzymatic work. I am indebted to Dr. N. Diding for valuable help in arranging the bacteriological tests, to Dr. B. Hagström for kind cooperation in carrying out the fertilisation experiments, and to Dr. K.-B. Augustinsson for carrying out the estimations of cholinesterase activity.

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Zur Kenntnis der Arylborsäuren. VII* Komplexbildung zwischen Phenylborsäure und Fruktose

Von KURT TORSSELL

Mit 1 Figur im Text

Einleitung

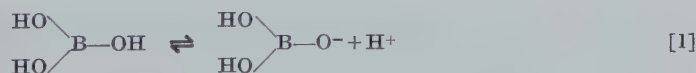
Es ist seit langem bekannt, dass Polyalkohole die Säurestärke einer Borsäurelösung erhöhen, was auf einer Komplexbildung zwischen Polyalkohol und Borsäure beruht (1). In Gegenwart von Mannit und Phenolphthalein als Indikator kann man Borsäure mit Lauge titrimetrisch bestimmen. Dies trifft auch für Phenylborsäure zu (2).

Da Fruktose die Säurestärke der Phenylborsäure beträchtlich erhöht, wurde dieser Zucker für unsere Untersuchungen über Form und Verhalten der Komplexe in wässriger Lösung gewählt. Der Mannit-Phenylborsäurekomplex ist in Wasser ziemlich schwerlöslich und fällt bei höheren Konzentrationen der Komponenten aus. Es hat sich herausgestellt, dass er aus 3 Mol Phenylborsäure pro Mol Mannit besteht (3), aber wahrscheinlich gibt es in der Lösung auch Komplexe, enthaltend 1 oder 2 Phenylborsäure pro Mol Mannit. Diese Untersuchung wurde ferner durchgeführt, um eine Auffassung über das komplexbildende Vermögen der Phenylborsäure im Verhältnis zu dem der Borsäure zu erhalten, denn daraus lassen sich Schlüsse über die pflanzenphysiologischen Wirkungen der Borsäure bzw. Phenylborsäure ziehen (4). Die Komplexkonstanten des Fruktose-Borsäuresystems sind früher gemessen worden (5).

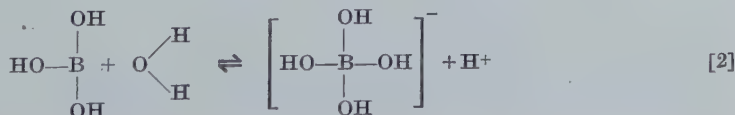
Das Verhalten der Borsäuren in wässriger Lösung

Die Dissoziation der Borsäure kann grundsätzlich auf zwei verschiedenen Wegen verlaufen.

1. Ein Proton wird von einer Hydroxylgruppe abgespalten:



2. Wasseranlagerung:

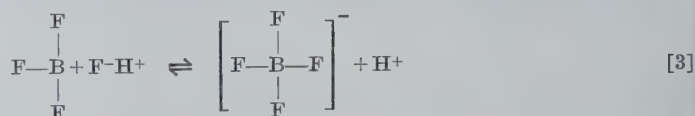


* VI. *Physiol. Plantarum*, 9 (1956) 652.

wobei Bor in seine koordinativ vierwertige Form übergeht. Dieses Gebilde muss völlig dissoziiert sein.

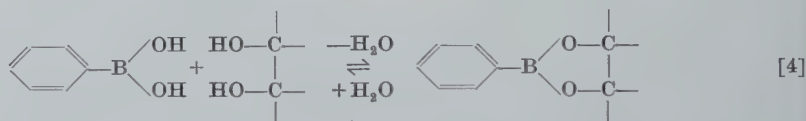
Das Ramanspektrum des Borations in wässriger Lösung zeigt, dass das Anion eine tetraedrische Symmetrie hat. Die Lage der Linien stimmt nicht mit der Struktur von H_2BO_3^- überein (6). Durch Röntgenuntersuchungen einiger fester Borate ist weiter festgestellt worden, dass das Boratom von vier Sauerstoffatomen umhüllt ist (7).

Das dreiwertige Bor hat das Bestreben, seine Oktettlücke aufzufüllen, was aus vielen Beispielen hervorgeht.

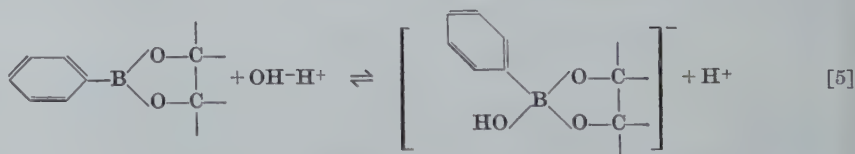


Diese Reaktion ist analog mit der Dissoziation der Borsäure nach [2]. Die bisherigen experimentellen Befunde deuten also darauf hin, dass die Dissoziation der Borsäure nach dem zweiten Mechanismus verläuft.

Diese Arbeit bringt nun ein neues Argument für diese Auffassung, unter der Voraussetzung, dass das, was für Phenylborsäure gilt, auch für Borsäure zutrifft. Zusatz von Fruktose zu einer Phenylborsäurelösung erniedrigt den pH-Wert beträchtlich. Es bildet sich dabei ein zyklischer Ester:



der keine Hydroxylgruppen mehr enthält. Wäre jetzt der erste Mechanismus der vorherrschende oder einzig mögliche, könnte keine Erhöhung der Acidität eintreten; sie würde vielmehr erniedrigt oder überhaupt nicht beeinflusst werden. Die Erhöhung der Säurestärke kann nur nach dem zweiten Mechanismus erklärt werden. Der Phenylborsäureester addiert ein Hydroxylion und gleichzeitig wird ein Proton frei:



Die Borsäure und Arylborsäuren können nicht als Säuren im Brönstedtschen Sinne betrachtet werden; sie sind Lewis-Säuren, d.h. sie haben das Bestreben ein Elektronenpaar zu addieren.

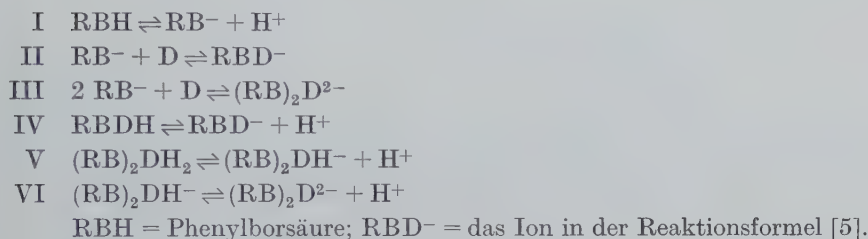
Wenn die Acidität einer Borsäure- oder Phenylborsäurelösung von Polyalkoholen erhöht wird, bilden sich also Ester (Komplexe), die eine grössere Neigung haben, Hydroxylionen anzulagern, als die Borsäure und Phenylborsäuren selbst. Das geht um so leichter, je ärmer das Bor an Elektronen ist. In Borsäureestern (B-O-C-Bindung) sind die freien Elektronenpaare des Sauerstoffs am Sauerstoff fester verankert als in Borsäure (B-O-H-Bindung), d.h. das Boratom der Borsäureester

ist elektronenärmer als das der Borsäure. Daraus lässt sich der Schluss ziehen, dass die Ester stärkere Säuren sind, als die freien Säuren, was mit den experimentellen Befunden übereinstimmt. Polyalkohole, die nicht die Säurestärke der Borsäuren beeinflussen, sollten nach diesen Betrachtungen überhaupt keine Komplexe bilden, obwohl man sich denken könnte, dass undissoziierte Komplexe entstehen können.

Gleichgewichte zwischen Fruktose und Phenylborsäure

Phenylborsäure kann wegen der Phenylgruppe nur ein Molekül eines Polyalkohols binden. Dabei sind nur ringförmige Komplexe in Betracht gezogen worden. Es ist dagegen denkbar, dass der Polyalkohol 2 oder höchstens 3 Phenylborsäurereste anlagern kann.

Es stellen sich folgende Gleichgewichte ein:



Aus den Gleichgewichten ergeben sich folgende Gleichungen:

$$1 \quad \frac{[\text{H}^+][\text{RB}^-]}{[\text{RBH}]} = K_s$$

$$2 \quad \frac{[\text{RBD}^-]}{[\text{RB}^-][\text{D}]} = K_1$$

$$3 \quad \frac{[(\text{RB})_2\text{D}^{2-}]}{[\text{D}][\text{RB}^-]^2} = K_2$$

$$4 \quad \frac{[\text{H}^+][\text{RBD}^-]}{[\text{RBDH}]} = K_3$$

$$5 \quad \frac{[\text{H}^+][(\text{RB})_2\text{D}^{2-}]}{[(\text{RB})_2\text{DH}^-]} = K_4$$

Ferner gilt:

$$6 \quad [\text{H}^+] + [\text{Na}^+] = [\text{RB}^-] + [\text{RBD}^-] + 2[(\text{RB})_2\text{D}^{2-}] + [(\text{RB})_2\text{DH}^-] + [\text{OH}^-]$$

$$7 \quad [\text{RBH}_{\text{tot}}] = [\text{RBH}] + [\text{RB}^-] + [\text{RBD}^-] + [\text{RBDH}] + 2[(\text{RB})_2\text{D}^{2-}] + 2[(\text{RB})_2\text{DH}^-] + 2[(\text{RB})_2\text{DH}_2]$$

$$8 \quad [\text{D}_{\text{tot}}] = [\text{D}] + [\text{RBD}^-] + [\text{RBDH}] + [(\text{RB})_2\text{D}^{2-}] + [(\text{RB})_2\text{DH}^-] + [(\text{RB})_2\text{DH}_2]$$

In einer Lösung, wo $[\text{RBH}_{\text{tot}}] \sim 0,1 \text{ M}$ und $[\text{D}] \sim 1 \text{ M}$, ist $[\text{H}^+] \sim 10^{-3} \text{ M}$ und $[\text{RBDH}]$ kann höchstens den Wert $= [\text{RBH}_{\text{tot}}]$ erreichen. Nehmen wir an, dass nur dieser Komplex entsteht, so findet man, dass die Dissoziationskonstante der RBDH grösser als 10^{-6} ist und zwar mit grösster Wahrscheinlichkeit um mehrere Zehnerpotenzen, denn undissoziierte RBDH konnte bei den Messungen nicht nachgewiesen werden. Bei pH-Werten > 5 kann man völlig von undissoziierten Komplexen absehen. Die Gleichungen 7 und 8 lassen sich folgendermassen vereinfachen:

$$7a \quad [\text{RBH}_{\text{tot}}] = [\text{RBH}] + [\text{H}^+] + [\text{Na}^+]$$

$$8a \quad [\text{D}_{\text{tot}}] = [\text{D}] + [\text{H}^+] + [\text{Na}^+]$$

$$[\text{OH}^-] \ll [\text{RBD}^-] \text{ oder } [\text{RB}^-]$$

Hieraus lassen sich $[\text{RBH}]$ und $[\text{D}]$ berechnen. Aus 1, 2, 3 und 6 ergibt sich:

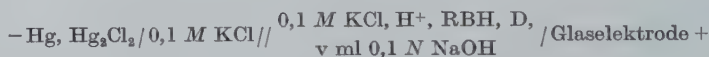
$$9 \quad \frac{([\text{H}^+] + [\text{Na}^+]) [\text{H}^+] - K_s [\text{RBH}]}{K_s [\text{RBH}] [\text{D}]} = K_1 + 2 K_2 [\text{RB}^-]$$

Setzt man wachsende Mengen Lauge zu einer Phenylborsäure-Fruktoselösung hinzu, verändert man $[\text{RB}^-]$. Würde man nun das linke Glied der Formel ($= A$ in der Tabelle) auf der Y -Achse auftragen und $[\text{BD}^-]$ auf der X -Achse, dann würde man eine Gerade erhalten, deren Neigung K_2 darstellt.

Emk-Messungen

Die Gleichgewichtskonstanten wurden in $0,1 \text{ M}$ KCl gemessen, um die Aktivitätsfaktoren konstant halten zu können. Die Konstanten gelten somit bei dieser Ionenstärke.

$[\text{H}^+]$ wurde durch Messung der Emk der folgenden Kette erhalten:



E° erhält man durch Messung der Emk bei einer bekannten $[\text{H}^+]$ und $[\text{H}^+]$ ergibt sich aus der Gleichung:

$$-\log [\text{H}^+] = \frac{E - E^\circ}{59,16}$$

Apparatur: Potentiometer, von Radiometer, Kopenhagen. Die Messungen wurden in einem Wasserthermostaten bei $25^\circ \pm 0,1$ durchgeführt.

Chemikalien: Kaliumchlorid, p.a. Merck, Darmstadt; *d*-Fruktose, C.P. Special, Pfanstiell Chemical Co, Waukegan, USA. Phenylborsäure (8) aus Benzol umkristallisiert. Sie kam dabei in ihrer anhydrischen Form heraus; Wasser mit Kohlendioxydschutz destilliert.

Um Kohlendioxyd aus dem Titrationsgefäss auszuschliessen, wurde gereinigter Stickstoff langsam durch die Lösung geleitet. Er sorgt gleichzeitig für die Umrührung.

Eine abgemessene Lösung von bekanntem Phenylborsäure- und Fruktosegehalt wurde mit $0,1 \text{ N}$ Natronlauge titriert. Die Emk wurde nach jedem Zusatz gemessen. $[\text{RBH}_{\text{tot}}]$ und $[\text{D}_{\text{tot}}]$ wurde in den verschiedenen Messreihen variiert. Vor dem Beginn der Messung wurde die Fruktoselösung so lange sich selbst überlassen, dass die Mutarotation der Fruktose mit Sicherheit beendet war.

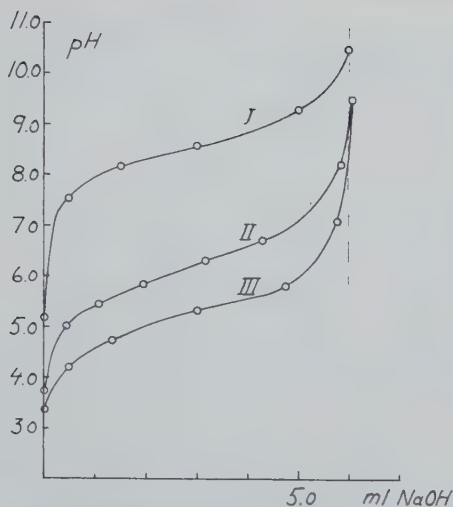


Fig. 1. Neutralisationsverlauf von Phenylborsäure. I, 20 ml $3,00 \times 10^{-2}$ M RBH wurden mit 0,1 N NaOH titriert; II, $3,00 \times 10^{-2}$ M RBH + $6,7 \times 10^{-2}$ M Fruktose; III, $3,00 \times 10^{-2}$ M RBH + $4,4 \times 10^{-1}$ M Fruktose.

Besprechung

In Tabelle 2 und 3 sind einige typische Messungen bei zwei verschiedenen Fruktosekonzentrationen wiedergegeben. Man sieht daraus, dass das linke Glied der Gleichung 9 ($= A$), wie erwartet, unabhängig von $[D]$ ist, d. h. Phenylborsäure bindet nur ein Fruktosemolekül. Weiter ist A von $[RBH]$ und $[RB^-]$ fast unabhängig. Bei steigendem Laugezusatz, d. h. bei steigender $[RB^-]$ sinkt der A -Wert sehr wenig. Erwartungsgemäss sollte er mit wachsender $[RB^-]$ steigen. Fruktose kann also nur ein Phenylborsäuremolekül binden. Der A -Wert ist dann gleich K_1 , das aus verschiedenen Versuchsserien gleich $5,2 \times 10^3$ gefunden wurde. Die entsprechende Konstante für Borsäure ist früher gemessen, $K_1 = 6,9 \times 10^2$, (5). Der Phenylborsäurewert ist etwa 7mal grösser, was mit den theoretischen Überlegungen (4) im Einklang steht.

Die Dissoziationskonstante der Phenylborsäure wurde bei derselben Ionenstärke 0,1 M gemessen und gleich $2,3_8 \times 10^{-9}$ gefunden. Branch, Yabroff und Bettman (2) geben $1,37 \times 10^{-9}$ (in reinem Wasser) an. Tabelle 1 zeigt eine Messreihe.

Tabelle 1. Bestimmung der Dissoziationskonstante der Phenylborsäure. 25,00 ml $2,994 \times 10^{-2}$ M RBH wurden mit 0,1 N NaOH titriert. $E^0 = -40,2$ mV.

ml NaOH 0,1000 N	[RBD] $\times 10^2$ M	[RB ⁻] $\times 10^2$	Emk mV	$-\log [H^+]$	$[H^+]$ $\times 10^8$	$K_s \times 10^9$
0,00			267,7	5,205	625	1,30
0,35	2,814	0,138	392,5	7,314	4,85	2,38
1,03	2,479	0,396	422,7	7,825	1,50	2,39
1,51	2,253	0,570	434,8	8,029	0,935	2,37
2,91	1,639	1,043	458,7	8,433	0,369	2,35
4,00	1,202	1,379	474,2	8,695	0,202	2,32
4,45	1,030	1,511	480,1	8,795	0,160	2,35
5,01	0,825	1,669	488,7	8,940	0,115	2,33

Tabelle 2. Titrierung von 30,00 ml $1,227 \times 10^{-2}$ M RBH und $4,454 \times 10^{-1}$ M Fruktose mit 0,1 N NaOH. $E^0 = -40,2$ mV.

ml NaOH 0,1000 N	[Na ⁺] × 10 ³	[D]	[RBH] × 10 ²	Emk mV	−log [H ⁺]	[H ⁺] × 10 ⁵	A × 10 ^{−3}
0,00		0,4454	1,197	167,8	3,516	30,5	(7,17)
0,38	1,25	0,439	1,087	216,3	4,336	4,62	5,27
0,80	2,60	0,431	0,935	239,1	4,721	1,90	5,17
1,22	3,91	0,424	0,788	253,7	4,968	1,08	5,29
2,10	6,54	0,410	0,493	280,0	5,412	0,387	5,27
2,48	7,63	0,404	0,370	292,5	5,624	0,238	5,11
3,08	9,31	0,395	0,182	317,6	6,048	0,0895	4,88

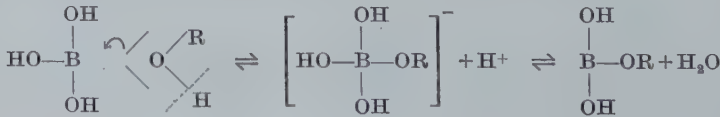
Tabelle 3. Titrierung von 20,00 ml $2,994 \times 10^{-2}$ M RBH und $6,697 \times 10^{-2}$ M Fruktose mit 0,1 N NaOH. $E^0 = -40,2$ mV.

ml NaOH 0,1000 N	[Na ⁺] × 10 ³	[D] × 10 ²	[RBH] × 10 ²	Emk mV	−log [H ⁺]	[H ⁺] × 10 ⁶	A × 10 ^{−3}
0,00			2,977	183,0	3,773	168,7	(6,02)
0,45	2,20	6,33	2,708	256,9	5,022	9,51	5,19
1,05	4,99	5,86	2,345	284,0	5,480	3,31	5,03
1,95	8,88	5,21	1,839	308,1	5,887	1,30	5,04
2,55	11,31	4,81	1,524	321,4	6,112	0,772	5,00
4,30	17,70	3,74	0,694	361,2	6,788	0,163	4,67

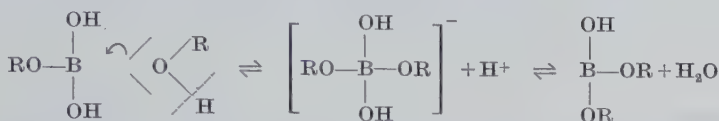
Figur 1 stellt den Neutralisationsverlauf von Phenylborsäure I und von Phenylborsäure + Fruktose II und III dar. Man sieht daraus, dass Phenylborsäure sich in Gegenwart von Fruktose und Phenolphthalein als Indikator titrimetrisch bestimmen lässt. Der Neutralisationspunkt liegt beim pH 8,5–9,0. Die Fruktosekonzentration muss dabei grösser sein als die Phenylborsäurekonzentration.

Mechanismus der Borsäureveresterung

Wir haben gesehen, wie Borsäure oder Phenylborsäure befähigt sind, ein weiteres Sauerstoffatom anzulagern unter Bildung eines Anions. Der Veresterungsvorgang lässt sich nach diesem Schema veranschaulichen (siehe auch Wiberg und Sütterlin (9)). Das Elektronenpaar des Alkoholsauerstoffs wird in die Elektronenlücke des Bors hineingezogen und gleichzeitig wird ein Proton ausgestossen:



Ein weiteres Alkoholmolekül wird jetzt angelagert.



usw.

Zusammenfassung

Die Komplexbildung zwischen Phenylborsäure und Fruktose wurde untersucht. Die Gleichgewichtskonstante der Reaktion: $\text{RB}^{-} + \text{D} \rightleftharpoons \text{RBD}^{-}$ wurde gemessen; $K_1 = 5,2 \times 10^3$. Komplexe, die mehr als 1 Mol Phenylborsäure pro Mol Fruktose enthalten, konnten nicht nachgewiesen werden.

Die Borsäure und Phenylborsäure sind Säuren im Lewisschen Sinne. Sie „dissoziieren“ durch Anlagerung von einem OH^{-} . Gleichzeitig wird ein Proton frei.

Der Mechanismus der Borsäureveresterung wird besprochen.

Für die sprachliche Durchsicht danke ich Dr. med. G. von Ehrenstein.

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Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

Isotope effect of hydrogen in aromatic sulphonation. II¹

Temperature dependence

By ULLA BERGLUND-LARSSON

With 3 figures in the text

As reported in earlier papers [1, 2], it was found that there was an isotope effect in aromatic sulphonation, with the value of $k_T/k_H = 0.55 \pm 0.09$ at 0°C, for the couple tritium-protium. This value corresponds to an unusually weak isotope effect if the splitting off of the hydrogen is the rate determining stage, a value of 0.2 or less being in better agreement with other experiments. A possible explanation of this value would be that the reaction proceeds in two stages with both stages rate-determining. Under this condition all the rate constants of the different stages are integral parts of the total expression for the reaction rate.

In general the high temperature limiting value for a ratio like k_T/k_H will be the square root of the inversed ratio between the reduced masses along the reaction coordinate [3]. This value could be presumed to be of the order $1/\sqrt{3}$ to 1 for k_T/k_H . The value already found is close to the lower one of these limits. It could hardly be assumed, however, that the high temperature limit should be reached already at about room temperature, where for ordinary chemical bond oscillators $h\nu \gg kT$. Thus the agreement is probably fortuitous, and it may still be worth while to investigate the temperature dependence. If the mechanism discussed earlier [2] holds, the variation could be expected to be extremely complex, as each constant in the expression for the total velocity has its own temperature coefficient. The purpose of the present investigation was to test the reaction scheme proposed in 1953 and for that reason the temperature dependence of the isotope effect was studied both for the couple tritium-protium and for deuterium-protium.

The experiments were carried out mainly in the same manner as used in 1953, with rather dilute solutions of fuming sulphuric acid and bromobenzene in nitrobenzene. The yield of the reaction was found by determining the amount of bromobenzene remaining in the nitrobenzene by infra-red spectrometric measurements.

To study the extent to which sulphone formation took place during a sulphonation reaction, the technique of isotope dilution was used.

To check that no spontaneous hydrogen exchange took place during the sulphonation, ordinary bromobenzene was sulphonated with oleum containing tritium, under the same conditions as those used in the main experiments. The remaining bromoben-

¹ Ref. [2] is considered as Part I.

zene was practically free from tritium. This shows that the sulphonation under these conditions is much more rapid than the hydrogen exchange. The latter is, however, predominant in aqueous sulphuric acid.

Experimental

Tritium.—The tritium used was obtained from the Atomic Energy Research Establishment, Harwell.

Measurement of tritium.—The measurements of tritium were carried out mainly in the same manner as used in earlier investigations [4, 1a, 2]. All organic compounds were burned to water and the water reduced to hydrogen which was used for inside counting in a Geiger-Müller tube. The accuracy of the determinations was now within $\pm 1\%$. This was made possible by thermostating the whole apparatus and by eliminating errors due to isotope separation in the reduction of the water to hydrogen.¹ By pumping with a mercury bulb the pressure of the unreduced sample was reduced to a value of less than 4×10^{-4} mm Hg. Thus the reduction was brought very close to completion, which makes any separation effect insignificant.

The unit of the specific activity used throughout the investigations is referred to the Geiger-Müller tube used and corresponds to about 250 disintegrations per millimole of hydrogen per minute. The corrections for the natural decay of tritium were made assuming its half-life to be 12.5 years.

The organic substances were burned in oxygen over red hot cupric oxide in one tube and passed through a second tube containing heated silver wool and a second layer of cupric oxide.

In some later experiments it was found that the samples poisoned the Geiger-Müller tube used, presumably due to some traces of bromine or of a bromine compound passing the hot cupric oxide and silver wool. According to Scott [5] bromine should be completely absorbed by silver wool at 180–200°C in quantitative determinations of organic compounds. It was found, however, that even under these conditions the Geiger-Müller tube was poisoned. A trial was then made using a layer of silver wool 20 cm long with a temperature gradient along the tube from 750°C to 50°C. Not even this prevented the poisoning of the Geiger-Müller tubes. The sensitivity to poisoning of the tubes varied considerably, probably depending upon different purity of the copper mantle or of the tungsten wire. No results were taken from poisoned Geiger-Müller tubes.

Deuterium.—The deuterium used was obtained from Norsk Hydro.

Measurement of deuterium.—The measurements of deuterium were carried out directly on the bromobenzene with the aid of a Perkin Elmer infra-red spectrometer, model 112, at the wave length of 11.85μ (845 cm^{-1}) where bromobenzene-4-*d* has a strong absorption band in contrast to ordinary bromobenzene. The thickness of the cell was 0.10 mm and its windows were of sodium chloride. The slit width used was 0.400 mm. In all infra-red measurements the whole absorption band studied was recorded in order to eliminate a possible error in the wavelength setting.

The small amount of nitrobenzene which was still left in the bromobenzene after the distillations was determined at the wavelength of 12.64μ (790 cm^{-1}) in the experiments with bromobenzene-4-*d*. The same cell was used as above and the slit width

¹ The new apparatus for the determination of tritium was constructed by Fil. kand. Stig Olsson at this institute.

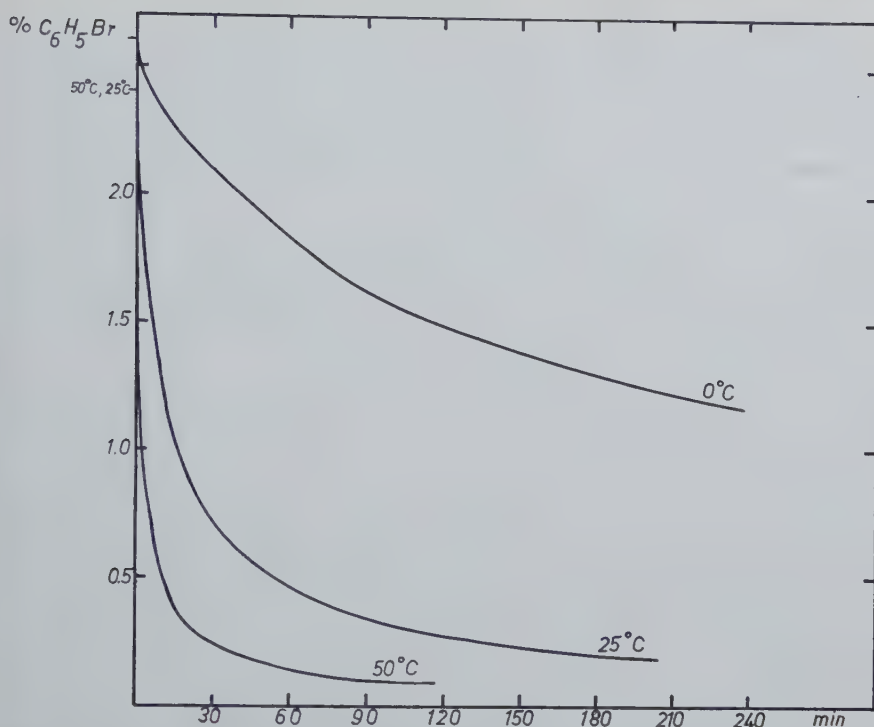


Fig. 1. Per cent by weight of remaining bromobenzene in sulphonation reactions at different temperatures, plotted against time. (The curves start at the marks outside the frame.)

was now 0.500 mm. The nitrobenzene, which has also a strong absorption band at 11.72μ (853 cm^{-1}) caused some overlapping on to the absorption band of bromobenzene at 11.85μ . The values of bromobenzene-4-d were corrected for this overlapping.

Determination of the yield.—After the reaction was quenched in icewater, the yield was obtained by determining the amount of bromobenzene remaining in the nitrobenzene by infra-red measurements. Equal reaction mixtures were used in all experiments and the reaction rate for a certain temperature is under these conditions a function of time only. In order to know at which time the reaction should be quenched some preliminary experiments were carried out at the temperatures 0°C , 25°C and 50°C . Samples were withdrawn from the reaction mixtures at different times and the amounts of remaining bromobenzene were plotted against time, Fig. 1.

The out of plane C-H bending, both of ordinary bromobenzene and of bromobenzene-4-d, gives an absorption band at 13.60μ (735 cm^{-1}), which in the polar solvent nitrobenzene is displaced to 13.50μ (740 cm^{-1}). The total amount of bromobenzene in the nitrobenzene was determined at the wavelength of 13.50μ . The cell was the same as that used above and the slit width was 1.150 mm. For the concentration range observed, i.e. up to 3 per cent by weight, Beer's law was obeyed.

Preparation of bromobenzene-4-t.—Bromobenzene-4-t was prepared from *p*-dibromobenzene by means of the decomposition of the corresponding Grignard compound with an ether solution of $\text{HCl} + \text{H}_2\text{O}$ containing tritium [1 b].

For a check of the isomeric purity of the preparation of bromobenzene-4-*t*, a sample was converted into 2,4-dinitrobromobenzene [1c]. The preparation contained less than 0.8 % of the tritium in positions other than para. The value is calculated on the assumption of a contamination by the ortho isomer. If meta is assumed the value should be halved. The purification of the *p*-dibromobenzene seems to be decisive.

The radioactive bromobenzene was diluted with a suitable amount of inactive bromobenzene (Merck & Co., U.S.A., anal. reagent).

Preparation of bromobenzene-4-d.—Bromobenzene-4-*d* was prepared from *p*-dibromobenzene by means of the decomposition of the corresponding Grignard compound with D₂O. The amount of D₂O used was three times the theoretical one. When about one quarter of the D₂O had been added, most of the ether was distilled off. To the concentrated ether solution the remaining D₂O was added and the reaction mixture was allowed to stand for three days before it was worked up.

The isomeric purity of the bromobenzene-4-*d* was the same or better than that of bromobenzene-4-*t*, since the *p*-dibromobenzene used was the same as in the tritium preparation, but crystallized once more.

As no extreme precautions were taken to exclude atmospheric moisture during the preparation of the bromobenzene-4-*d*, some ordinary bromobenzene was also formed. In order to determine the absolute deuterium content of the bromobenzene-4-*d*, a sample was burned to water, the water diluted with a suitable amount of ordinary distilled water and the deuterium determined by infra-red spectroscopy [6]. The deuterium content of the undiluted water was found to be 18.55 % which corresponds to a bromobenzene-4-*d* concentration of 86.0 %.

The bromobenzene-4-*d* was diluted with ordinary bromobenzene (Merck & Co., U.S.A., anal. reagent) until the concentration of the deuterium compound was about 1 %.

Fuming sulphuric acid.—The fuming sulphuric acid used was Baker's analyzed, 15–18 % SO₃ and 20–23 % SO₃. In order to check the SO₃ content, known portions of water were added to a sample and, from the freezing point curve obtained, the amount of water equivalent to the free SO₃ was determined (pure H₂SO₄ corresponding to maximum freezing point) [7].

Nitrobenzene.—The nitrobenzene used as solvent (E. Merck, "with determined refractive exponent") was dried with calcium chloride and distilled under reduced pressure.

Sulphonation of bromobenzene-4-t at 0°C.—Weighed amounts of nitrobenzene (250 ml) and oleum (15 ml) were introduced into a flask and cooled to 0°C. 5.0 ml of bromobenzene-4-*t* at 0°C was added, the mixture being placed in an ice-bath. The solution was magnetically stirred during the reaction. After a suitable time, the reaction was stopped by pouring the solution into ice water, and during vigorous stirring, was made alkaline with sodium hydroxide. The phases were separated. The nitrobenzene phase was further washed with sodium carbonate and water and dried with calcium chloride. The yield of the reaction was now determined from the remaining amount of bromobenzene in the nitrobenzene phase as found with the infra-red spectrometer. The bromobenzene was distilled off under reduced pressure in a vacuum-jacketed Widmer column (with spiral). It was distilled twice more under reduced pressure in an ordinary Claisen flask. The boiling point and the index of refraction of the final preparation agreed well with that of the pure bromobenzene.

Sulphonation of bromobenzene-4-t at 25°C and 50°C.—The same quantities of nitrobenzene and oleum as used above were weighed out and introduced into a double

Table 1. Check experiments against hydrogen exchange.

s = spec. act. averaged over all hydrogens in the system.

s_1 = average spec. act. of bromobenzene hydrogens, the activity first being averaged over oleum and 1 of the initial bromobenzene hydrogens.

s_3 = same, but with 3 initial bromobenzene hydrogens.

s_5 = same, but with 5 initial bromobenzene hydrogens.

$s_{C_6H_5Br}$ and $s_{C_6H_5NO_2}$ = observed final average spec. act. of hydrogen in resp. molecule.

Exp.	Temp.	Time min.	Strength of oleum % free SO_3	Yield %	Calculated				Observed	
					s	s_1	s_3	s_5	$s_{C_6H_5Br}$	$s_{C_6H_5NO_2}$
1	50°C	4	15-18	75.9	14.1	68	172	249	1.2	0.11
2	25°C	7	15-18	57.4	—	65	166	242	1.7	—

jacketted flask and thermostat water was passed through the external jacket. The temperature of the reaction vessel was kept constant to within $\pm 0.1^\circ C$. The same amount of bromobenzene as above was thermostated and added and the reaction mixture was shaken vigorously. After a suitable time the reaction was stopped and treated in the same manner as in the experiments carried out at $0^\circ C$.

Sulphonation of bromobenzene-4-d at $0^\circ C$.—The same quantities as used above were weighed out, introduced into a flask and cooled to $0^\circ C$. A weighed quantity (5 ml) of bromobenzene-4-d in an ampoule was thermostated, introduced into the same flask and the ampoule broken. The solution was magnetically stirred during the reaction. After a suitable time the reaction was stopped, the phases treated as above and the yield determined. The bromobenzene was distilled off under reduced pressure in a vacuum jacketted Widmer column (with spiral) and distilled once again in a Podbielniak column. In spite of the fact that the boiling point and the index of refraction agreed with the data of pure bromobenzene, it was found that the bromobenzene was contaminated with some nitrobenzene. The amount of the contaminating nitrobenzene was determined by infra-red spectrometric measurements.

Sulphonation of bromobenzene-4-d at $25^\circ C$ and $50^\circ C$.—The same quantities of nitrobenzene and oleum as used above were weighed out and introduced into a double jacketted flask and thermostated. About the same amount of bromobenzene as used above was weighed out in an ampoule and thermostated. The ampoule was introduced into the flask and broken. The flask was shaken vigorously. After a suitable time the reaction was stopped and treated as in the experiments with bromobenzene-4-d at $0^\circ C$.

Check experiments against hydrogen exchange. In two sulphonations of ordinary bromobenzene at $25^\circ C$ and $50^\circ C$ respectively, 10 mg of water containing tritium were added to the reaction mixture. After a suitable time the reaction was stopped and treated in the same manner as in the experiments of bromobenzene-4-t carried out at $25^\circ C$ and $50^\circ C$. The data are given in Table 1.

Check experiments against sulphone formation.—In order to study to what extent sulphone formation takes place during a sulphonation reaction, the yield was determined in two different ways. On one hand the yield was determined as the amount of bromobenzene consumed, on the other hand it was determined, as the amount of bromobenzenesulphonic acid formed. If no side-reactions take place, and all the bromobenzene gives sulphonic acid, the two methods would give the same result.

Table 2. Check experiment against sulphone formation.

Exp.	Temp.	Time min.	Strength of oleum % free SO ₃	Yield	
				Determined by infra-red spectroscopy	Determined by isotope dilution
3	50°C	3.75	15-18	58.8	56.1

If, however, any sulphone was formed, more bromobenzene should have been consumed than bromobenzenesulphonic acid formed.

To study how much bromobenzenesulphonic acid is formed, the technique of isotope dilution was used. A known amount of the sodium salt of bromobenzene-3-*t*-4-sulphonic acid was added to the ice-water in which the sulphonation was to be quenched. The reaction mixture was added to the ice-water and made alkaline with sodium hydroxide. The mixture was stirred vigorously for half a day. The aqueous phase was then filtered, made just neutral with sulphuric acid and evaporated to dryness. The residue was powdered and extracted three times with boiling absolute ethanol. The extract was filtered and evaporated to dryness. The residue was recrystallised from the same solvent, extracted with boiling ether to remove sulphone contaminations and dried over phosphorus pentoxide under reduced pressure.

The sodium salt of the bromobenzenesulphonic acid which had been purified as above was burned in the same manner as the bromobenzene and the tritium activity of the water was determined. From the known amount of salt added and from the specific activities of added and final salt, the amount of the sodium salt of the bromobenzenesulphonic acid formed could be calculated.

The data are given in Table 2. The error in the yield determination by the technique of isotope dilution is $\pm 3\%$ and that of the infra-red measurement $\pm 1\%$.

Results

If the concentration of the ordinary bromobenzene is denoted by i , and that of bromobenzene containing heavy hydrogen by a (initial concentrations i_0 and a_0) the following equation is valid [1d]:

$$\frac{a}{a_0} = \left(\frac{i}{i_0} \right)^\beta, \quad (1)$$

$$\frac{a}{i} \cdot \frac{i_0}{a_0} = \frac{y}{y_0} = (1-x)^{-(1-\beta)}, \quad (2)$$

where y_0 denotes the ratio of the concentration of the heavy hydrogen compound to the ordinary compound at zero time, and y this ratio when the fraction x of the bromobenzene has reacted. β is the ratio k_T/k_H or k_D/k_H , k_T , k_D and k_H being the specific rates of the tritium compound, the deuterium compound and the ordinary compound. The results may conveniently be represented in a double-logarithmic diagram:

$$\log \frac{y}{y_0} = -(1 - \beta) \log (1 - x). \quad (3)$$

If the isotope effect is independent of the yield, as it should be, points corresponding to different yields should be situated on the same straight line through the origin. β is obtained from the slope of this line. When tracer amounts are used and the heavy molecules are very few in comparison with the ordinary ones, the ratio a/i is measured. In the case of tritium, the specific activity is used as a measure of this ratio. In the case of deuterium, however, the heavy molecules cannot be neglected in comparison with the ordinary ones and the ratio $a/(a + i)$ is measured in this case instead of a/i .

$$\text{If} \quad x = \frac{i_0 - i}{i_0}, \quad 1 - x = \frac{i}{i_0}$$

we have according to equation (1)

$$\begin{aligned} \frac{a}{a + i} &= \frac{a_0 \left(\frac{i}{i_0} \right)^\beta}{a_0 \left(\frac{i}{i_0} \right)^\beta + i} = \frac{a_0 (1 - x)^\beta}{a_0 (1 - x)^\beta + i_0 (1 - x)} = \frac{\frac{a_0}{i_0} (1 - x)^\beta}{\frac{a_0}{i_0} (1 - x)^\beta + (1 - x)}, \\ \frac{\frac{a}{a + i}}{\frac{a_0}{a_0 + i_0}} &= \frac{y'}{y_0} = \frac{a_0 + i_0}{i_0} \frac{(1 - x)^\beta}{\frac{a_0}{i_0} (1 - x)^\beta + (1 - x)} \\ &= \frac{a_0 + i_0}{i_0} \frac{1}{1 + \frac{a_0}{i_0} (1 - x)^{\beta-1}} (1 - x)^{\beta-1} = f(x, \beta) (1 - x)^{-(1-\beta)}. \end{aligned} \quad (4)$$

In equation (4) y'_0 denotes the heavy hydrogen compound fraction in the total amount of the compound at the time origin and y' the same fraction, when the fraction x of the ordinary bromobenzene has reacted.

If $a_0 \ll i_0$ we get equation (2).

In the present case $a_0/i_0 = 0.009$.

When y'/y'_0 goes from 1 to 1.4 as it does in the deuterium experiments, $f(x, \beta)$ goes from 1 to 0.996.

The yield is defined as $(i_0 - i)/i_0 = x$, but in the deuterium experiments the ratio $[i_0 + a_0 - (i + a)]/(i_0 + a_0) = x'$ is measured, when determining the yield, as both kinds of molecules have the same absorption in the region concerned.

$$\frac{1 - x'}{1 - x} = \frac{i + a}{i_0 + a_0} \frac{i_0}{i} = \frac{1 + \frac{a}{i}}{1 + \frac{a_0}{i_0}} = g'(x).$$

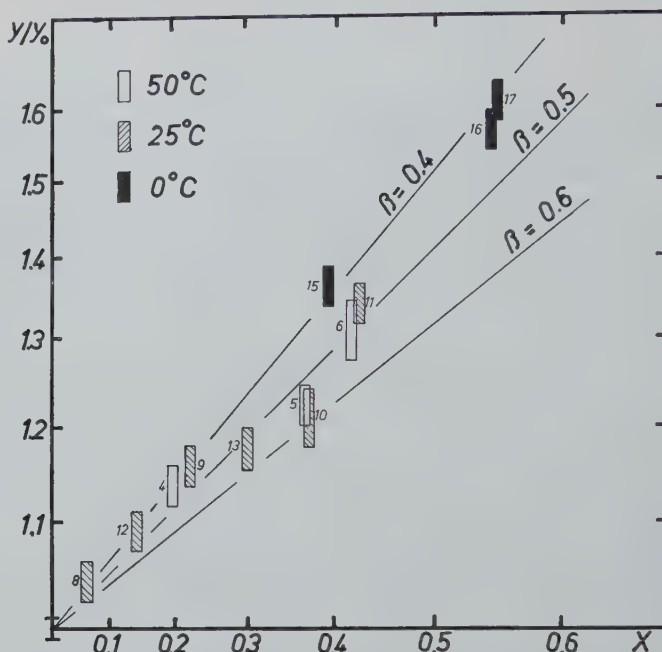


Fig. 2. Double-logarithmic representation of the relative change in specific activity (y/y_0) of remaining bromobenzene-4-*t* as a function of the sulphonated fraction (x). Cf. Table 3.

According to equation (4) we have

$$\frac{y'}{y_0} = f(x, \beta) \frac{1}{[g'(x)]^{\beta-1}} (1-x')^{\beta-1} = f(x, \beta) g(x, \beta) (1-x')^{-(1-\beta)}. \quad (5)$$

The highest value of a/i in these experiments is 0.014.

$g(x, \beta) = [g'(x)]^{1-\beta}$ goes from 1 to 1.004 when a/i goes from 0.01 to 0.014.

If equation (5) is used instead of equation (2) in the deuterium experiment, and $f(x, \beta)$ and $g(x, \beta)$ are put equal to unity, a systematic error in β is made. As $g(x, \beta)$ is always equal to or larger than unity, and $f(x, \beta)$ is equal to or less than unity, the errors are of the same magnitude and will counteract each other. Moreover, as the limits of error of the experiments are much larger than the small systematic errors involved, the latter will not influence the result.

The results from the sulphonation of bromobenzene-4-*t* are given in Fig. 2 and the condition under which the corresponding individual experiments were carried out are found in Table 3. As the boiling point and index of refraction of the bromobenzene recovered agreed with the values of pure bromobenzene, there was no suspicion that the bromobenzene was contaminated with nitrobenzene. Not until the infra-red determinations of the bromobenzene-4-*d* were carried out, was it proved that some traces of nitrobenzene might still be left in the bromobenzene. The highest nitrobenzene content which was ever found in the bromobenzene was 2 %. As the experiments with bromobenzene-4-*t* were carried out before the experiments with bromobenzene-4-*d*,

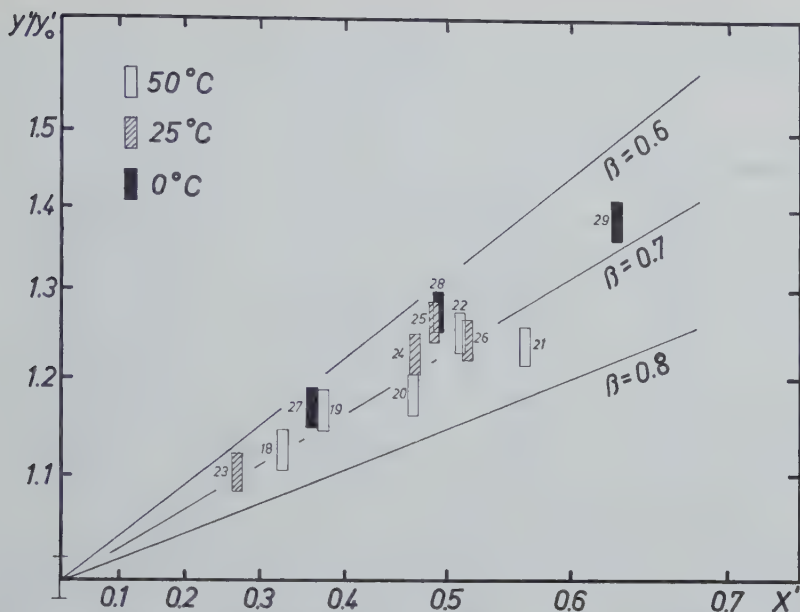


Fig. 3. Double-logarithmic representation of the relative change in the fraction of heavy molecules (y'/y'_0) of remaining bromobenzene-4-*d* as a function of the sulphonated fraction (x'). Cf. Table 4.

no corrections have been made in the tritium experiments for a possible dilution of the sample originating from the presence of nitrobenzene. The error of $\pm 2\%$, due to a possible dilution of the sample has been added to the limits of error along the ordinate. The limits of the total error along the ordinate are thus $+3\%$ and -1% , since a dilution of the sample causes an error in only one direction. The error along the abscissa (error in remaining amount of bromobenzene) is $\pm 1\%$.

The results from the sulphonation of bromobenzene-4-*d* are given in Fig. 3 and the corresponding conditions in Table 4. Corrections have been made for the dilution of the sample originating from the presence of nitrobenzene. The limits of error are those of the infra-red measurements, *i.e.* $\pm 2\%$ when determining the deuterium content, and $\pm 1\%$ in the determination of the remaining bromobenzene.

Discussion

The check experiments against hydrogen exchange (Table 1) show that the bromobenzene, as well as the solvent nitrobenzene, are practically stable towards exchange when oleum of the above strength is used. The error introduced, depending on a very small possible hydrogen exchange, is quite negligible in comparison with the limits of error of the experiments. The temperatures of the check experiments for bromobenzene were 25°C and 50°C , and for nitrobenzene 50°C . In a previous work [2] it was found that the bromobenzene and the solvent nitrobenzene were practically stable at 0°C . As almost no hydrogen exchange takes place with the solvent nitrobenzene at 50°C and 0°C there is no reason to assume it to take place at 25°C .

Table 3. Data of sulphonations of bromobenzene-4-*t*, cf. Fig. 2.

Exp.	Temp.	Time min.	Strength of oleum % free SO ₃	<i>n</i> _D of final bromobenzene
4	50°C	0.5	15-18	1.5593 (20°C)
5	50°C	1.0	15-18	1.5597 (20°C)
6	50°C	1.5	15-18	1.5596 (20°C)
7	50°C	3.75	15-18	1.5592 (20°C)
8	25°C	1.0	15-18	1.5596 (20°C)
9	25°C	3.0	15-18	1.5595 (20°C)
10	25°C	6.0	15-18	1.5591 (20°C)
11	25°C	9.0	15-18	1.5592 (20°C)
12	25°C	15.0	15-18	1.5592 (20°C)
13	25°C	0.75	20-23	1.5592 (20°C)
14	25°C	2.0	20-23	1.5592 (20°C)
15	0°C	60	15-18	1.5584 (23°C)
16	0°C	90	15-18	1.5570 (26°C)
17	0°C	182	15-18	1.5581 (23°C)

Table 4. Data of sulphonations of bromobenzene-4-*d*, cf. Fig. 3.

Exp.	Temp.	Time min.	Strength of oleum % free SO ₃	Remaining C ₆ H ₅ NO ₂ in per cent by volume
18	50°C	0.33	15-18	0.18
19	50°C	0.67	15-18	0.39
20	50°C	1.0	15-18	0.32
21	50°C	1.5	15-18	0.30
22	50°C	2.0	15-18	0.40
23	25°C	2.5	15-18	0.00
24	25°C	8.0	15-18	0.16
25	25°C	9.5	15-18	0.56
26	25°C	10	15-18	0.41
27	0°C	20	15-18	0.07
28	0°C	60	15-18	0.19
29	0°C	80	15-18	0.07

The fact, however, that a very small but real tritium content was found in the solvent nitrobenzene after a sulphonation reaction (experiment 1) shows that there is a small hydrogen exchange. As bromobenzene is more reactive than nitrobenzene, hydrogen exchange will occur more easily with bromobenzene and thus more tritium will be found in the bromobenzene than in the nitrobenzene. The tritium in the bromobenzene might also be due to the reversal of the sulphonation reaction. At the temperatures 25°C and 50°C it is, however, impossible to decide whether the slight tritium content in the bromobenzene is due to exchange or reversal of the sulphonation reaction, because the possible reverse reaction has not proceeded to any appreciable extent and a stationary state has not yet been reached (*cf.* Fig. 1). At 0°C, however, it was possible to investigate a reaction mixture, in which the reaction had come to an approximate standstill. It could be concluded that this stopping was not due to the operation of a reverse reaction[2]. At the higher temperatures a similar investigation was impossible for experimental reasons.

The check experiment against sulphone formation showed that appreciable sulphone formation does not take place. Since the proportion of sulphone formation is always favoured by increased temperature and since none is formed at 50°C, there is no reason to assume that it takes place at 25°C and 0°C.

In the experiments with bromobenzene-4-*t* it is found that the points corresponding to 0°C are situated slightly below the line representing the β -value 0.4. The points corresponding to 25°C and 50°C are situated below the points at 0°C, quite near the line with a β -value of 0.5. There is no significant difference between the points corresponding to 25°C and 50°C. The new β -value found at 0°C falls a little below the lower limit of that obtained in 1953. The small deviation between the old and the new β -values could be due to the appreciable uncertainty in the determination of the yield by titration in the early experiments.

In the experiments with bromobenzene-4-*d*, the tendency is that the points corresponding to 0°C are situated a little above the points corresponding to 25°C, which in turn are situated a little above the points corresponding to 50°C. The corresponding β -values are: at 0°C, 0.67; at 25°C, 0.70; and at 50°C, 0.73. This is, however, only a tendency and the change of the β -values is too small to be determined accurately with the present method and within the present temperature region.

There is no obvious trend with the fraction of the bromobenzene converted. The different anhydride concentrations used caused no change of the positions of the points in the experiments with bromobenzene-4-*t*.

The isotope effects obtained from the tritium and deuterium experiments agree very well with each other. They are also in very good agreement with the result of Brand [8]. In his sulphonation of nitrobenzene-*d*₅ in strong oleum (30–40 % anhydride), he obtained an isotope effect of $k_D/k_H = 2/3$.

There are two possible explanations of an isotope effect. The first is the pure S_E2 or S_E3 replacement reaction, where the π -electrons of the benzene nucleus do not take any part in the formation of the σ -bonds, although they must be more or less polarized. The other is the two-stage mechanism, where two of the π -electrons are used for the addition of an electrophilic species, thus forming the intermediate. If the formation of the intermediate is rate determining there would be no isotope effect, but if the splitting off of the hydrogen is rate-determining, such an effect will occur. Both the direct replacement reaction and the second alternative of the two-stage mechanism would, however, give a much stronger isotope effect than that obtained.

The low isotope effect for the sulphonation reaction was explained in 1953 [2] by assuming the reaction to proceed according to a two-stage mechanism with the second stage only partly rate-determining. As the total expression for the velocity will be built up from the rate constants of the different stages and as each constant has its own temperature coefficient, the temperature dependence of the isotope effect will be extremely complex. The temperature coefficient for the isotope effect might also be zero for certain intervals, in which the temperature coefficients of the constants compensate each other.

Hence, the weak isotope effect obtained and its small variation with temperature, is in favour of the reaction scheme proposed in 1953.

Interesting results in favour of the two-stage mechanism of electrophilic aromatic substitution have also been obtained by Zollinger in his investigation of the isotope effect in the azo coupling [9].

The common absorption band of ordinary bromobenzene and bromobenzene-4-*d*

at 13.60μ (735 cm^{-1}) which originates from the C-H out-of-plane bending is displaced to a shorter wavelength (higher frequency) in the polar solvent nitrobenzene. According to Kross, Fassel and Margoshes [10] the sp^2 bond of the carbon can, in a benzenoid system, enter the field of the π -electrons, forming a hybrid having some sp^3 character. If the normal π -electron clouds of the benzene nucleus are depleted by an electrophilic substituent the possibility of hybridisation is reduced and hence a higher frequency of the C-H out-of-plane bending will occur.

The shift of the C-H out-of-plane bending frequency of bromobenzene to a higher frequency when the polar solvent nitrobenzene is used might be explained in the following way: bromobenzene is a dipole with the bromine negative and the benzene nucleus positive. The polarity of the solvent nitrobenzene facilitates further polarisation of the bromobenzene. The electron density of the benzene nucleus will thus decrease, and hence a shift to a higher frequency will occur.

Summary

For the determination of the temperature dependence of the isotope effect of sulphonation, sulphonation of bromobenzene containing either tracer amounts of bromobenzene-4-*t* or small amounts of bromobenzene-4-*d* has been carried out with oleum in nitrobenzene as solvent at the temperatures 0°C , 25°C and 50°C . The very small variation of the isotope effect with temperature supports the reaction scheme proposed in 1953.

The author is indebted to the Head of the Institute, Docent Lars Melander, for his kind interest in this investigation and many valuable discussions. The Swedish Natural Science Research Council has contributed to the acquirement of the infra-red spectrographic equipment.

Nobel Institute of Chemistry, Stockholm 50, Sweden, June 29, 1956.

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Separation of *cis-trans* isomers by ion-exchange chromatography

4-chloro-*cis*-cinnamic acid and 2,4-dichloro-*cis*-cinnamic acid

By SVEN LINDENFORS

With 6 figures in the text

Ever since the discovery of auxins, many attempts have been made to find the structural requirements for auxin activity. In 1938 Koepfli, Thimann and Went (1) put forward the following general requirements for growth activity: an unsaturated ring, an acid side-chain and a particular spatial arrangement between the ring and the side-chain. The authors did not, however, express the spatial arrangement more exactly, but in 1944 Veldstra (2) concluded that the configuration must be such that the acid side-chain can be out of the plane of the unsaturated ring nucleus.

Model studies of α,β -unsaturated acids of cinnamic acid type show that in the *trans* form the side-chain is in the plane of the ring, while in the *cis* form it is most probable that the side-chain is out of the plane of the ring. Already in 1935 Haagen Smit and Went (3) found that *cis*-cinnamic acid was an auxin and that *trans*-cinnamic acid was inactive. In 1951 Overbeek (4) reported that *trans*-cinnamic acid was an anti-auxin.

A great deal of work has been done by Professor Fredga and Dr. Matell on *optical isomers*, among other things in connection with their different influence on auxin activity. It therefore seemed interesting to investigate some *geometrical isomers* which may be expected to have properties as growth regulators (5).

The most stable form of the isomers of cinnamic acid type is the *trans* form which is always obtained when the usual methods for preparing α,β -unsaturated acids are used. To convert the *trans* form into the *cis* form essentially two methods have been used, the first is by the action of different catalysts and the other, which is more universal in the case of substituted cinnamic acids, is by irradiation with a strong ultraviolet light source (6). The irradiation of the *trans* acid solution leads to a photochemical equilibrium which is in the favour of the *trans* form. By changing wavelength, solvent and temperature it might be possible to displace the equilibrium more in favour of the *cis* form. This will be discussed in a later paper.

In most cases one of the greatest problems in dealing with *cis-trans* isomers is to separate the two forms. In the case of substituted cinnamic acids Nivard (6) and others have isolated the *cis* form over the aniline salt in different solvents but it is a rather time-consuming process to get a perfectly pure *cis* acid by that method. The present author has found that 4-chloro-*cis*-cinnamic acid, which was first pre-

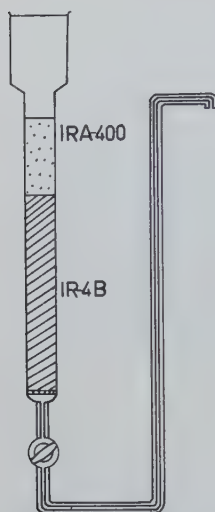
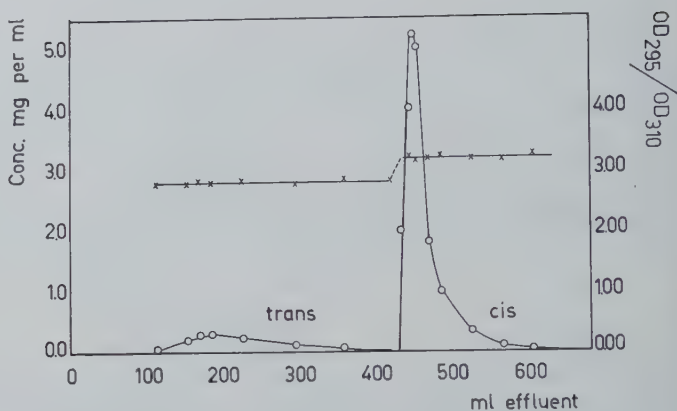


Fig. 1.

Fig. 2. Separation of *cis*- and *trans*-4-chloro-cinnamic acid.

pared by Nivard (6), is not quite stable when stored unprotected and exposed to daylight in the laboratory. When stored in glass bottles or best in the dark the *cis* form seems to be quite stable. The author has worked out a method for separating *cis*- and *trans*-4-chloro-cinnamic acid by ion-exchange chromatography. The same method was also used for preparing 2,4-dichloro-*cis*-cinnamic acid, which has not previously been described.

The biological examination of the *cis-trans* isomers is being carried out by Professor B. Åberg who will describe these experiments in a separate publication.

Davies (7) has shown that for the separation of two weak acids by elution the best separation will in most cases be achieved when the pH of the solution applied to the resin is 1–2 units lower than the value of $\frac{1}{2}(pK_a + pK'_a)$ where K_a and K'_a are the dissociation constants of the two acids. In the case of substituted cinnamic acids the *trans* acid is, with few exceptions, the weaker acid and should then be preferentially eluted. The substituted cinnamic acids are slightly soluble in water and therefore the author has used ethanol–water mixtures in which the acids are more soluble. The strongly basic resin Amberlit IRA-400 in the hydroxyl form adsorbes *cis*- and *trans*-4-chloro-cinnamic acid both in 50 and 75 % (v/v) ethanol. Acetate-buffer itself does not elute the acids but the chloride ion does. Therefore mixtures of acetate-buffer varying both in concentration and acidity from 0.005 to 0.2 *M* and pH 3.7 to 4.5 respectively, together with sodium chloride in varying concentration up to 0.3 *M* have been used. There seems to be not only pure ion-exchange but also strong attraction between the acid molecules and the resin. There seems also to be a partition chromatography effect which makes the difference in acidity insufficient in itself to bring about a good separation on that resin. Displacement chromatography with a concentration gradient and the chloride ion as a displacing agent has been tried without great success. The author therefore tried a new resin, the weakly basic resin Amberlit IR-4B. The acids were not adsorbed in such a narrow band at the top of the column as in the case of Amberlit IRA-400 and on eluting with 0.004 *M* hydro-

chloric acid in 75 % (v/v) ethanol the *trans* acid was displaced first. To get a better separation the two resins mentioned above were combined in the same column, the one above the other, as seen in Fig. 1. The *cis* form can then be eluted with a mixture of 0.4 acetic acid and 0.3 *M* sodium chloride in 75 % (v/v) ethanol. As seen from Fig. 2 the *trans* acid is eluted rather slowly at first, after which the principal part of the *cis* form is easily eluted. To use the method on a preparative scale most of the *trans* form is therefore eliminated before separation by dissolving the acid-mixture after irradiation in boiling water in which the *cis* form is more soluble than the *trans* form. The *trans* acid is thereby reduced to only a few per cent of the total weight but it is precisely these small quantities which are difficultly eliminated by other methods. The author has prepared pure 4-chloro-*cis*-cinnamic acid on a 1 g scale by this method on a rather small column (40 × 1 cm). The 2,4-dichloro-*cis*-cinnamic acid has been prepared in exactly the same way. The elution has been followed both qualitatively and quantitatively by measuring the optical densities (O.D.) at 2950 and 3100 Å. A constant value for the ratio $O.D._{2950}/O.D._{3100}$ indicates the presence of a single component which is seen in Fig. 2.

The infrared spectra of *cis*- and *trans*-4-chloro-cinnamic acid and *cis*- and *trans*-2,4-dichloro-cinnamic acid have been investigated because, as shown by Rasmussen and Brattain (8), such spectra are of great value in elucidating problems of *cis* and *trans* structure. *trans*-Ethylenic double bonds give rise to a medium to strong band at 10.10–10.36 μ . This has been shown by Kilpatrick and Pitzer (9) to be due to the hydrogen atoms which are out of plane at the double bond. As seen from Figs. 3 and 5 the *trans*-ethylenic bond for 4-chloro-*trans*-cinnamic acid and 2,4-dichloro-*trans*-cinnamic acid lies at 10.15 and 10.17 μ . Figs. 4 and 6 show that in the corresponding *cis* isomers this band is not present. In 4-chloro-*cis*-cinnamic acid a very weak band is seen at about 10.14 μ but this band cannot be due to 4-chloro-*trans*-cinnamic acid as the bands in the latter at 11.45 and 14.0 μ are totally eliminated in the *cis* form.

Experimental

4-Chloro-trans-cinnamic acid.—The acid was synthesised according to Doebner's method for preparing α,β -unsaturated acids. The yield (80 %) is higher than that of Perkin's reaction. The acid was recrystallized twice from 85 % formic acid and finally from ethanol, m.p. 248–250°. Different reports on the melting point, varying between 242–250°, are found in the literature (6, 10, 11, 12). All melting points in this paper have been determined according to the microscopical methods of Kofler and Kofler (13). The apparatus used was a *Reichert* hot stage microscope.

Anal. Subst., 136.20 mg; 7.66 ml of 0.0976 *N* NaOH.
Equ. wt. Calc. 182.6. Found 182.2.

4-Chloro-cis-cinnamic acid.—15 g 4-chloro-*trans*-cinnamic acid was neutralized with 1 *N* NaOH and the pH brought to about 11 by an excess of alkali and finally diluted to 225 ml with water. The solution was irradiated in an open dish with a quartz lamp, Philips HPK 125 W, in a special aluminium reflector at a distance of 10 cm from the surface of the solution. The irradiation time was 40 hours and the temperature in the solution was kept between 15–20° by means of a glass-spiral in which cold water was circulated. The solution was acidified with 2 *N* HCl and the acid-mixture filtered off and dried at room temperature in the dark. The *cis* acid

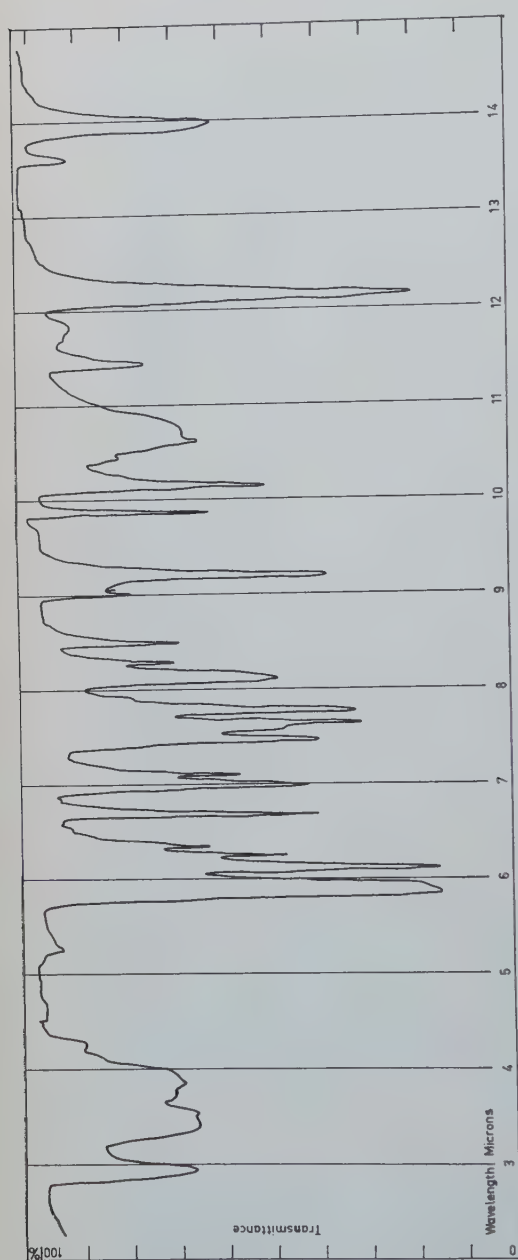
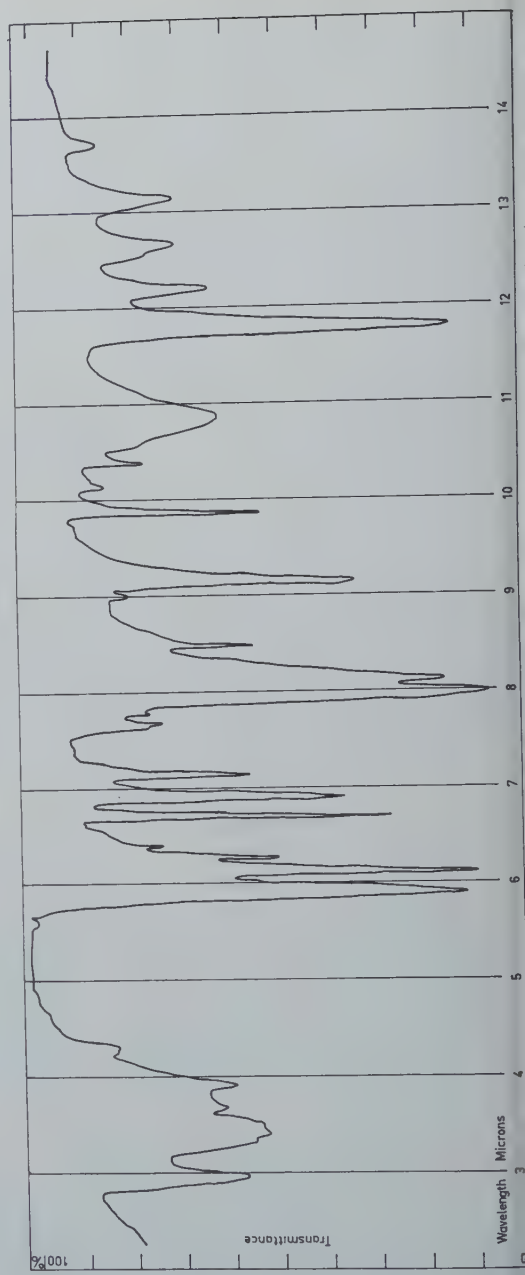


Fig. 3. IR absorption curve of 4-chloro-*trans*-cinnamic acid.



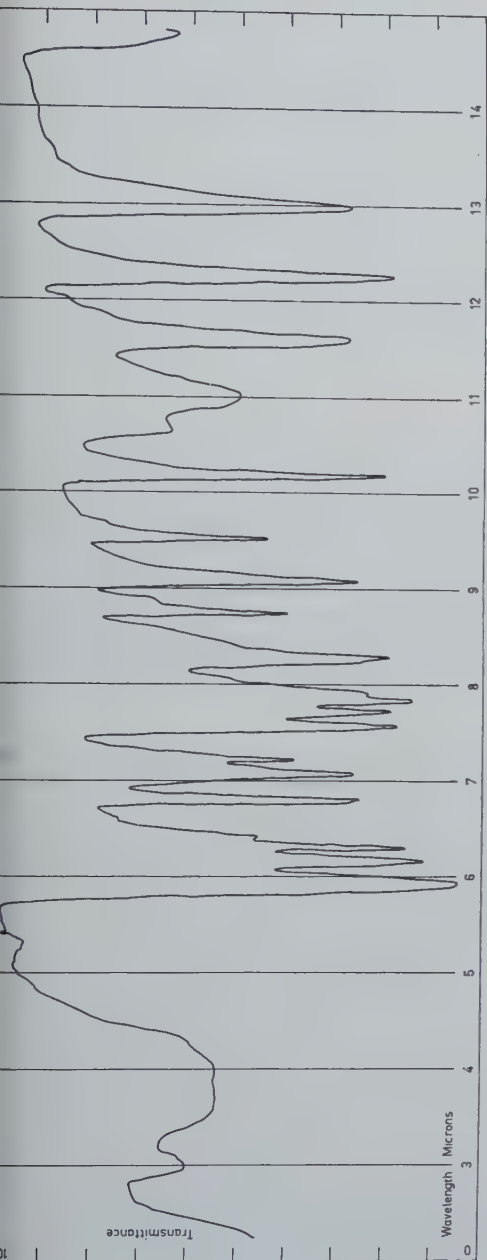


Fig. 5. IR absorption curve of 2,4-dichloro-*trans*-cinnamic acid.

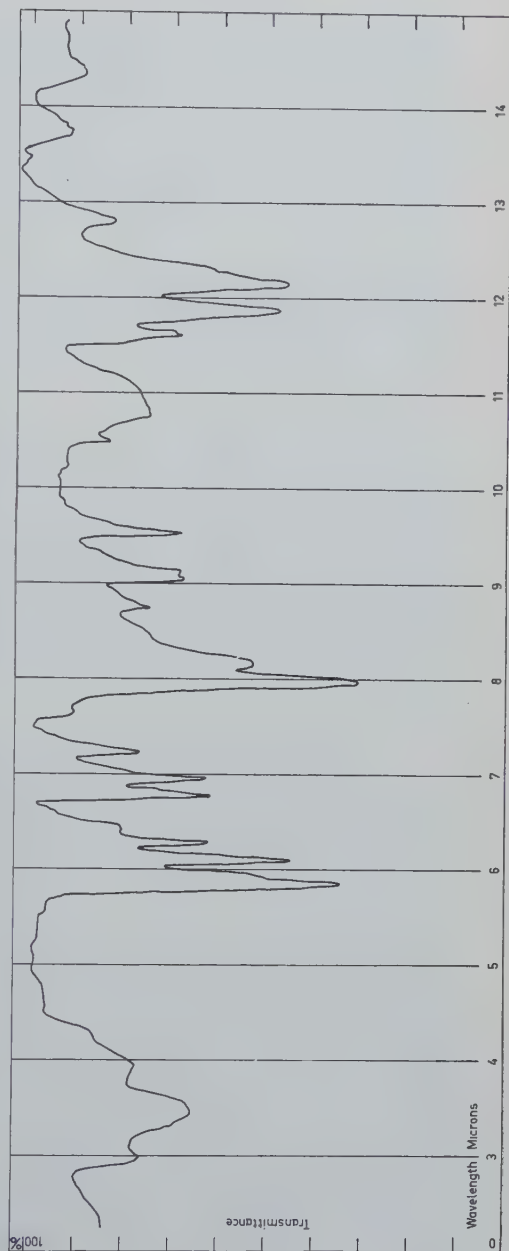


Fig. 6. IR absorption curve of 2,4-dichloro-*cis*-cinnamic acid.

was extracted with 4×100 ml of boiling water, cooled, filtered off and dried at room temperature in the dark. Yield 2.6 g, m.p. $107-120^\circ$. The *cis* acid was then purified by ion-exchange chromatography which will be discussed in detail below. An acid with m.p. $113.8-116.2^\circ$ was obtained. The melting point taken in the normal way, viz. in a capillary tube, showed an interval of about 1° . Nivard (6) gives the m.p. 115° for 4-chloro-*cis*-cinnamic acid.

Anal. Subst., 94.31 mg; 5.28 ml of 0.0976 *N* NaOH.
Equ. wt. Calc. 182.6. Found 183.0.

2,4-Dichloro-trans-cinnamic acid.—The acid was synthesised according to Doebner. The acid was recrystallized twice from glacial acetic acid and once from ethanol. Yield 75 %. M.p. $233.0-234.5^\circ$.

Anal. Subst., 209.2 mg; 9.85 ml of 0.0976 *N* NaOH.
Equ. wt. Calc. 217.05. Found 217.6.

2,4-Dichloro-cis-cinnamic acid.—10 g 2,4-dichloro-*trans*-cinnamic acid was neutralized with 2-aminoethanol, the pH brought to about 10 by adding an excess of amine and the solution finally diluted to 200 ml with water. The sodium salt is too slightly soluble to give yields of practical importance. The solution was irradiated for 40 hours in the same apparatus as described above, acidified with 2 *N* HCl and airdried in the dark. The mixture of *cis-trans* acids thus obtained was extracted with 4×100 ml of boiling water, filtered off and dried at room temperature in the dark. Yield 0.8 g, m.p. $120-130^\circ$. The *cis* acid was then purified by ion-exchange chromatography as described in the next section. An acid with melting point $131.5-133.9^\circ$ was obtained.

Anal. Subst., 24.30 mg; Co_2 , 44.11 mg; H_2O , 5.84 mg.
Subst., 64.30 mg; 6.02 ml of 0.0976 *N* NaOH (combustion according to Grote-Krekeler).
Subst., 107.3 mg; 5.05 ml of 0.0976 *N* NaOH.

$\text{C}_9\text{H}_6\text{O}_2\text{Cl}_2$ (217.05) Calc. C 49.80; H 2.79; Cl 32.67; Equ. wt. 217.05.
Found. C 49.51; H 2.69; Cl 32.40; Equ. wt. 217.7.

Separation of *cis-trans* isomers by ion-exchange chromatography

The resins used were Amberlit IR-4B and Amberlit IRA-400, the particle size range in both cases being 60–100 mesh. Before use the resins were treated with *N*-sodium hydroxide, *N*-hydrochloric acid and again with *N*-sodium hydroxide in all cases in 75 % (v/v) ethanol to remove any soluble matter. In the experiment the result of which is shown in Fig. 2 the solution contained more *trans* acid (20 %) than is usually obtained when the *cis* acid is extracted with boiling water. This solution was specially prepared in order to show more clearly the behaviour of the acids on the resin column. After filling, the column (13×1 cm IR-4B and 5×1 cm IRA-400) is washed with 75 % ethanol until the eluate is about 0.02 *M* with respect to sodium hydroxide. The last traces of alkali need not be washed out to get a good separation. A solution of 40 mg 4-chloro-*trans*-cinnamic acid and 160 mg of 4-chloro-*cis*-cinnamic acid in 45 ml 75 % ethanol was passed through the column at about 20 ml per hour. The acids are adsorbed at the top of the Amberlit IRA-400 and the resin thereby changes its colour from brown to yellow which makes it easy to see that the column is not overloaded. The *trans* acid is then eluted with 0.004 *M* hydrochloric

acid in 75 % ethanol at a rate of 15–20 ml per hour. The *cis* acid is eluted with a solution containing 0.4 *M* acetic acid and 0.3 *M* sodium chloride in 75 % ethanol. The fractions were collected with an automatic fraction collector. The *trans* acid fractions were diluted with the same solution by which the *cis* acid was eluted and measured with a Beckman Model DU Spectrophotometer, in order to make it possible to compare the ratio $O.D._{2950}/O.D._{3100}$ for the two acids. The ethanol is driven off *in vacuo* from the *cis*-acid fractions and the acid is recrystallized once from water in order to remove inorganic matter and dissolved resin. The 2.4-dichloro-*cis*-cinnamic acid was purified in the same way.

A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prisms was used for the infrared absorption measurements. The acids were examined as solid pressed potassium bromide plates. The calibration of the absorption curves is correct to $\pm 0.01 \mu$ at about 10 μ .

Summary

A method for separation of *cis-trans* isomers by ion-exchange chromatography has been developed.

2.4-dichloro-*cis*-cinnamic acid, which is a new substance, has been prepared.

Infrared spectra of *cis*- and *trans*-4-chloro-cinnamic acid and *cis*- and *trans*-2.4-dichloro-cinnamic acid are given. It is shown that by the use of such spectra it is possible to determine the structure of *cis*- and *trans* compounds.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Professor Arne Fredga for valuable advice and discussions. The author is also indebted to Dr. Magnus Matell for suggesting the investigation and for interesting discussions. The infrared curves have been recorded by Dr. Andreas Rosenberg with technical assistance from Mr. Stig Bergvall. Finally I am indebted to Mr. Sixten Johansson for some help with the experimental work.

This investigation has been supported by Jordbrukets Forskningsråd to which acknowledgement is gratefully made.

Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, October 1956.

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Tryckt den 2 februari 1957

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A micro-scale classification and determination of sugar using the phloroglucinol reaction

By NILS OLOF LINDH

With 3 figures in the text

Von Euler and Hahn (1) have described a method for the determination of ribose in nucleic acid preparations by means of a colour reaction between the pentose and phloroglucinol. This method seems to be suitable also for analysis of glycogen, other glucose derivatives, fructose, mannose, galactose, arabinose, xylose, rhamnose and glucuronic acid. Though not dealt with in this work, ascorbic acid reacts like a pentose. Amino sugar and deoxyribose do not react with phloroglucinol.

The method permits a determination of small quantities of different sugars present in the same test, which is of great value in biochemical and physiological experiments, where the samples or tissue fractions available for examination usually are small. The method can be used in micro-analysis, which is of special value when the amount of sugar mixture is too small for chromatographic separation, or one wants to know the sugar content in a test, which is to be analysed in other respects.

Method

Reagents.—*Solution A* is composed of 0.1 % FeCl_3 (sicc.) in 1 part concentrated hydrochloric acid and 6 parts glacial acetic acid. *Solution B* contains 0.25 % phloroglucinol in 1 part concentrated hydrochloric acid, 2 parts glacial acetic acid and 1 part water.

Procedure.—1 part of the sugar solution to be analysed is mixed with 8 parts of solution A. The test is made in at least duplicate. The tubes are air-tightly closed and after that they are kept at 100°C for 50 min., during which treatment the sugar is converted to a furfural derivative. Then the tests are cooled to room temperature. Now 1 part of solution B is added to the tests. The tubes are again closed and shaken to homogeneity, after which they are allowed to stand for 20 minutes at room temperature. Finally one part of the tests is heated for exactly 4 minutes and the other part for 40 min. After this heating they are cooled in ice water, in order to stop the reaction. The coloration is read in a spectrophotometer against a similarly treated A-blank with B solution. When the original test solution is coloured or becomes coloured during heating, one must compensate with a test blank without phloroglucinol in B solution.

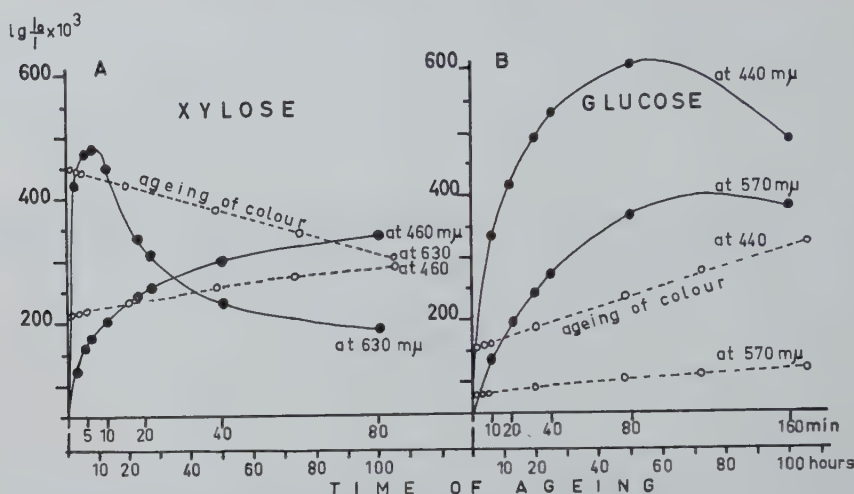


Fig. 1. A and B. Variation of optical density with the time of heating at 100°C (●—●); for xylose at 630 and 460 $m\mu$ and for glucose at 440 and 570 $m\mu$. ○-----○ the effect of storage at room temperature after 4 min. heating at 100°C.

The concentration of sugar in reaction mixture ought not to exceed 20 μg per ml, because the relation between concentration and light absorption can not be approximated to a straight line above that amount of sugar. If it turns out to be higher the original sugar solution must be diluted.

Von Euler and Hahn worked with comparatively large volumes; 1 ml sugar solution, 8 ml solution A and 1 ml solution B. The mean error, however, is only raised to $\pm 5\%$ if the total volume of reaction mixture is reduced to 0.5 ml (0.4 ml A + 0.05 ml test + 0.05 ml B) and the sugar concentration is 16.5 μM in glucose or 10.5 μM in xylose. The mean error is $\pm 1\%$ at 77 μM glucose and 49 μM xylose.

In order to prevent condensation of water in the reaction mixture and evaporation of hydrochloric and acetic acid, the test tube must be stoppered with glass or airtightly closed with a polyethylene film. Rubber or cork is not suitable. When working on a micro-scale it is better to use ampullae with a bulb and a long thin-walled narrow neck. The bulb facilitates the proper mixing and the long neck prevents undue warming of the reaction mixture. The ampullae are preferably filled with horizontal pipettes, which are tipped with capillaries ligated with polyethylene tubing. The capillary is bent to a siphon with the tip just below the level of the pipette. With this arrangement it is possible to make accurate pipetting, which is of the utmost importance since two of the reaction mixtures are coloured or become coloured during the heating at 100°C.

A standard sugar solution must be run simultaneously because the FeCl_3 solution is not quite stable. Its qualities change owing to both evaporation and oxygenation, which increases progressively as the storing vessel is emptied. A sealed and cold FeCl_3 solution does not change. The phloroglucinol solution is quite stable, if kept dark and cold. It does not matter if it should darken a little.

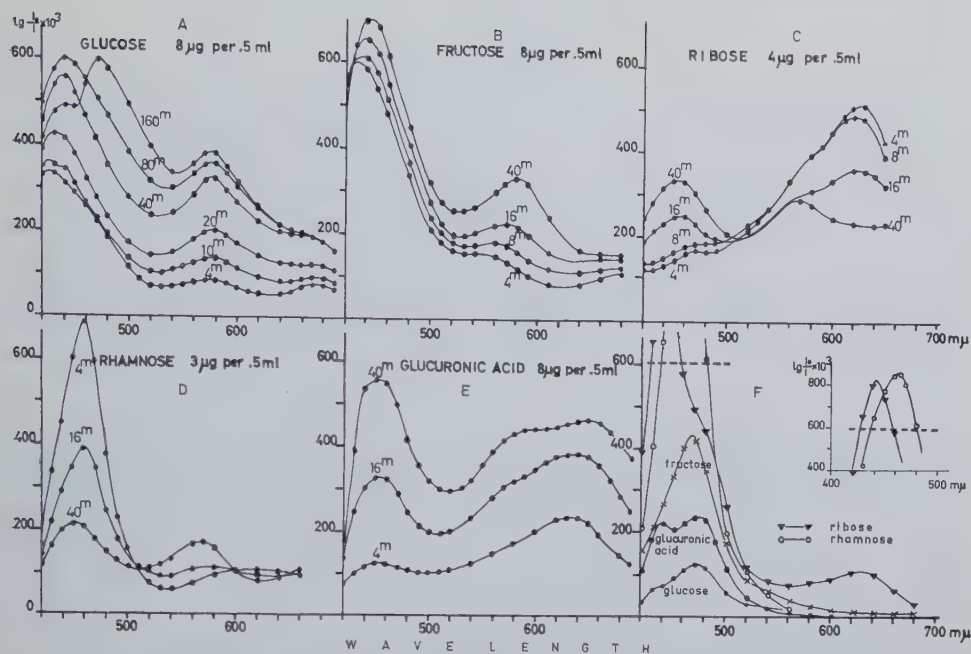


Fig. 2. A-E. Colour curves of glucose, fructose ($8 \mu\text{g}$ in each test); ribose, $4 \mu\text{g}$; rhamnose, $3 \mu\text{g}$; glucuronic acid, $8 \mu\text{g}$. The amount is examined in total volume of 0.5 ml reaction mixture. The time of heating at 100°C has been varied from 2 to 160 minutes.

F. Optical density of glucose, glucuronic acid, fructose, ribose and rhamnose 20 min. after addition of phloroglucinol without heating.

Results

Treated with phloroglucinol in FeCl_3 solution the different sugars give different coloration, as seen from Figs. 2 A-E. The coloration of galactose, mannose, xylose and arabinose resembles that of glucose and ribose respectively, but the molar extinctions are different. The coloration is dependent on the time of heating with phloroglucinol, see Figs. 1 and 2, and the concentration of FeCl_3 , see Table 1. All sugars give immediately after addition of phloroglucinol an intense yellow colour; absorption maximum at $440\text{--}460 \text{ m}\mu$, see Fig. 2 F. This primary coloration fades away and is replaced by other colours. The second and more stable coloration depends on the temperature-time factor, see Figs. 1 A and B. The pentoses, represented by xylose, reach a maximal extinction at $620\text{--}630 \text{ m}\mu$ after 4 to 6 min. Prolonged heating causes that extinction to vanish. If the reaction mixture is cooled in ice water immediately after the 4 min. heating and afterwards is allowed to stand at room temperature the extinction will decrease in any case (denoted as ageing in the figure) but at a speed, which is 2^8 times slower. The same is valid in the case of glucose, but here the maximal extinction at $440\text{--}450 \text{ m}\mu$ occurs after about 80 min. of heating at 100°C . After shorter heating, the ageing of colour at room temperature results in an increasing extinction. It is thus possible to reach a desired colour maximum with rather great accuracy simply by using suitable time and temperature.

The furfural derivatives of aldohexoses and uronic acids (Figs. 2 A and E) lose

Table 1.

For explanation see text.

m μ	Glucose		Fructose		Ribose		Glucuronic acid	
	0 % 4 ^m 40 ^m	0.2 % 4 ^m 40 ^m	0 % 4 ^m 40 ^m	0.2 % 4 ^m 40 ^m	0 % 4 ^m 40 ^m	0.2 % 4 ^m 40 ^m	0 % 4 ^m 40 ^m	0.2 % 4 ^m 40 ^m
430	262 925	234 234	496 1205	592 308	169 414	307 231	106 615	96 150
480	245 357	194 621	340 306	421 915	214 276	400 641	193 393	169 420
570	73 173	59 510	105 153	124 596	543 214	661 871	81 298	161 620
630	— 85	40 224	16 110	73 250	898 271	1025 921	86 403	243 712

their first coloration very rapidly and are slow in the formation of the second and more stable colour. The absorption maximum of aldohexoses is at 422 m μ after 4 min. of heating. The absorption peak is displaced to 437 m μ and even longer waves on prolonged heating. Glucuronic acid has an increasing "second" absorption at a constant wavelength, 450 m μ . Fructose retains the first coloration somewhat longer than aldohexoses, but on heating the second colour is developed more rapidly. The absorption peak, which is reached after 4 min., grows only a little on further heating simultaneously with the displacement of the peak from 427 to 440 m μ . At the same time as the first short wave peak develops a less specific absorption is formed at 560–580 m μ . The maximum of this absorption does not change wavelength in the case of glucose, mannose or galactose. In the case of fructose the peak shifts from 540 to 570 m μ during 4 to 40 min. of heating. Glucuronic acid gives the 570 m μ -peak first after about 40 min. of heating.

The first unspecific coloration of pentoses is more stable than that of fructose, but it disappears on heating. Instead they develop an absorption maximum at 620–630 m μ , which is reached after 4 to 6 min. at 100°C. Prolonged heating destroys this pentose specific coloration, which is replaced by a double-peak curve as in the case of hexoses.

The first developed colour with methylpentose vanishes very slowly, and after 16 min. of heating half the maximal extinction at 450 m μ is still left. Continued warming results in a double-peak curve like other sugars, but the short wave peak is continuously lowered.

Prolonged heating with phloroglucinol results in all sugars except methylpentose first in a further elevation of the 440 m μ absorption followed by declination (see Fig. 2 A, 160 min. heating). These changes are sugar-specific. The 570 m μ extinction is continuously raised on heating and is unspecific.

If the FeCl₃ solution is substituted with only hydrochloric-acetic acid 1:6 or the concentration of FeCl₃ is changed to 0.2 %, the general performance is the same, see Table 1. In the first case there is an improved light absorption at short wave lengths but lessened at the longer. If the concentration of FeCl₃ is raised the case will be the contrary, a weakened light absorption at 400–460 m μ and a strong and more stable absorption in the yellow and red region, which is clearly demonstrated by ribose. Methylpentose is independent of the concentration of FeCl₃. The higher FeCl₃ concentration also displaces the absorption peak towards longer waves. The same situation is encountered with an ageing FeCl₃ solution, which means that the mixture has been aerated and part of the solvent has evaporated.

Table 2.

For explanation see text.

Wavelength, mμ	420	430	440	450	460	470	480	500	520	540	560	570	580	600	620	630	650		
$\lg \frac{I_0}{I} \times 10^3$	4 ^m 40 ^m		264	351	404	470	518	464	335	208	170	188	230	254	274	301	328	321	288
			343	417	464	462	437	392	337	261	243	269	331	348	334	280	236	233	230

Since, as seen above, the investigated sugars all have their own speed of reaction with phloroglucinol and the molar extinction at different wave lengths is specific, it will be possible to determine an unknown sugar. In a mixture it is possible firstly to distinguish between the groups hexoses, pentoses, methylpentoses and uronic acids, and secondly, if the mixture is not too complex, to determine the sugars in a group and estimate their amount. In order to differentiate between the groups aldohexoses, fructoses, pentoses, methylpentoses and uronic acids one has to determine the molar extinction after 4 min. of heating at those wavelengths where specific peaks occur, namely 422 (aldoses), 427 (fructose), 460 (rhamnose), 540 (fructose), 580 (aldoses) and 630 (pentoses). After 4 min. of heating the absorption spectrum and the molar extinction is nearly alike within the different groups of sugar, but after 40 min. of heating there are rather marked differences in molar extinction of the different sugars in a group. Now the peaks are gathered at 440 (hexoses and pentoses), 450 (rhamnose), 570 (all sugars) and 660 (pentoses and uronic acids).

The absorption values of a triplex mixture made of 0.4 ml A, 0.05 ml sugar solution and 0.05 ml B are given in Table 2. The sugar mixture is composed of glucose, ribose

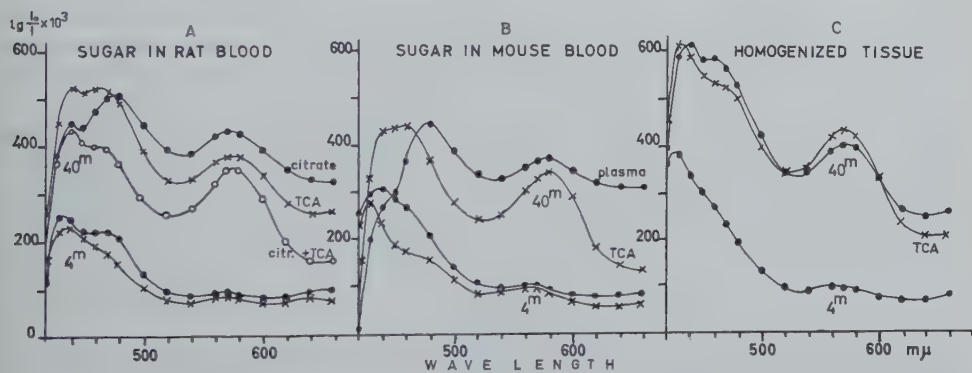


Fig. 3. A. Sugar analysis of citrate plasma from a rat (●—●), diluted 1:4 with 3 % sodium citrate, ×—× plasma treated with 4 volumes trichloroacetic acid (TCA), ○—○ TCA-treated citrate plasma. The 4 min. values correspond to 105 mg % "glucose" and the difference between citrate and TCA plasma corresponds to 100 mg % "glucose" in glucoprotein. The reaction with phloroglucinol is a little disturbed.

B. Corresponding analysis of true plasma from a mouse, diluted 1:4 with 0.8 % saline. The 4 min. value at 420 mμ corresponds to 135 mg % "glucose". Marked disturbances after 40 min. heating.

C. Sugar analysis of homogenate of flatworm tissues. The curve corresponds appr. to "glucose" and ribose in the proportions 120:1. The reaction is not disturbed by the protein present.

and rhamnose. The great light absorption value at 460 $m\mu$ indicates the presence of a methylpentose. The absorption at 620 $m\mu$ is typical for pentoses. At 420 $m\mu$ the light absorption is too great in relation to the expected sum of methylpentose and pentose, which reveals a hexose. On heating for 40 min. the eventual occurrence of uronic acid should be revealed by a levelled curve from 600 to 660 $m\mu$. Postulating that the mixture is composed of glucose, ribose and rhamnose, the absorption values at three wavelengths are required, for example 420, 580 and 630 $m\mu$. The extinction values $\times 10^3$ per μg sugar per 0.5 ml reaction mixture can be read in Figs. 2 A, C and D and are for glucose 41.8, 10.6 and 6.5; for ribose 30.0, 90.0 and 127; for rhamnose 50.3, 30.0 and 37.0. By using the formula below and three equations, it can be shown that the sugar solution in question contains 3 μg glucose, 2 μg ribose and 1.5 μg rhamnose. This means that the tested solution was 3.3×10^{-4} M in glucose, 2.6×10^{-4} M in ribose and 2.0×10^{-4} M in rhamnose.

$$E_G \cdot x + E_R \cdot y + E_{Rh} \cdot z = A_n;$$

E = specific (molar) extinction of glucose (G), ribose (R) and rhamnose (Rh) at wavelength n $m\mu$.

A = actual extinction at n $m\mu$ wavelength.

x, y, z = amount of sugar in μg per 0.5 ml reaction mixture.

In sugar determinations on rat and mouse blood, respectively (Figs. 3 A and B) the compensated 4 min. absorption curves are in good agreement with the known composition and concentration of blood sugar. The 40 min. curves, however, reveal great disturbances. The short wave absorption is too low and the resulting peak consequently displaced to a longer wave range. The sugar reaction in deproteinised plasma is not disturbed. Possibly the plasma protein carries an excess of oxygen and a certain oxidation of the phloroglucinol complex would be responsible for the deviation on prolonged heating. The same deflection of the absorption curve is obtained on 40 min. of heating, if one tries to determine an aldohexose in old oxygenated (aerated) $FeCl_3$ solution. If the plasma is stored at $+4^\circ C$ for 24 h the discoloration and the deviation of the absorption curve are markedly lower, which would mean that the oxygen was used up in breaking down special chromogenic substances.

In sugar determinations on a homogenate of flatworm tissues, see Fig. 3 C, the discoloration is negligibly small and the reaction with phloroglucinol is unaffected. The proportion between "glucose" and ribose is 120:1, calculated from the absorption values at 420 and 630 $m\mu$ after 4 min. heating. After 40 min. heating of TCA-treated material "glucose" is shown to be composed of 7.7 μg glucose-galactose and 4.3 μg fructose, presumably emanating from seminal fluid.

The sugar tests can be stored before the first 50 min. heating at $100^\circ C$ for a month in a deep-freeze without any change in quality.

Discussion

The method described permits rather large series to be run simultaneously because the handling is easy and the developed colour is nearly stable at room temperature. If the tests are kept in the refrigerator awaiting spectrophotometric analysis, there is no difference exceeding the mean error between the first and the last in a series of 6 tests. The influence of light on the phloroglucinol reaction has not been fully in-

vestigated. The phloroglucinol solution darkens on standing in daylight, but if the blank is treated as the sugar test this extra coloration is without importance.

The weaknesses of the method are the strong light absorption of FeCl_3 solution from $460 \text{ m}\mu$ to shorter waves and the colour of phloroglucinol, which increases with the time of heating. The method is thus dependent on the degree of accuracy in pipetting the different solutions.

With the described method it is fully possible to estimate all sugars except amino sugar and deoxyribose in a mixture, one only has to compare their specific (molar) extinction at a sufficient number of wavelengths. However, owing to the origin of the sugar mixture the composition is fairly well known and one has to register the total amount and the mutual distribution of sugar. The latter is made possible by prolonged heating with phloroglucinol. The differentiation within a group of sugars can be facilitated if the concentration of FeCl_3 is lowered in the case of hexoses and raised to 0.2 % in the case of pentoses.

The reaction between furfural derivatives and phloroglucinol is not disturbed by protein in general, which is demonstrated by addition of the above-mentioned sugar to the same protein solution. This specificity is reported by von Euler and Hahn. The disturbances occurring in sugar analysis of plasma are somewhat mysterious, because they are first revealed after about 10 min. heating at 100°C . Before that time added sugar is fully indicated but on longer heating the phloroglucinol-furfural complex is destroyed. This destruction is dependent on the time of storage of the plasma before analysis. Deproteinised plasma gives quite normal sugar reaction. Therefore one must assume that there is a hitherto unknown component of the protein which interrupts the sugar-specific condensation. Consequently, in the case of plasma one can only determine the total amount of sugar in the different groups—hexose, pentose, methylpentose and uronic acid—unless the plasma is deproteinized or hydrolyzed in alkali.

Von Euler and Hahn stated that phloroglucinol does not react with either amino sugar or deoxyribose. They suggested that deoxyribose would be destroyed during the first 50 min. heating in FeCl_3 solution. However, milder treatment does not result either in any coloration with phloroglucinol. One would think there should be a possibility of obtaining and fixing an unstable sugar by adding FeCl_3 and phloroglucinol to the test at once, like in the orcinol reaction. However, this treatment is without result in all sugars tested, because there are no furfural derivatives at the beginning and when they are formed the reaction ability of phloroglucinol is already destroyed. The amino sugar never forms any furfural derivative.

Summary

This work presents a method for micro-analysis of sugar solutions, elaborated from von Euler and Hahn (1). It is based on dehydration of sugar in 0.1 % FeCl_3 solution in concentrated hydrochloric-acetic acid (1:6) for 50 min., after which the carbohydrates give characteristic colour reactions with phloroglucinol. The individual sugars exhibit specific absorption spectra, which depend on the time of heating at 100°C and the time of storing after colour development. By heating for 4 min. the sugars are classified into groups as aldohexoses, ketohexoses, pentoses, methylpentoses and uronic acids. After 40 min. heating it is possible to differentiate between each member in a group. This differentiation is made easier in the case of hexoses, if one lowers the concentration of FeCl_3 when the pentoses are checked. Differentia-

tion of pentoses is better made in high concentration of FeCl_3 . The influence of protein on the phloroglucinol reaction is discussed. The carbohydrates investigated are glucose, fructose, galactose, mannose, arabinose, ribose, xylose, rhamnose and glucuronic acid.

Institution of Zoophysiology, University of Lund.

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Tryckt den 2 februari 1957

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Quantitative estimation of sialic acids

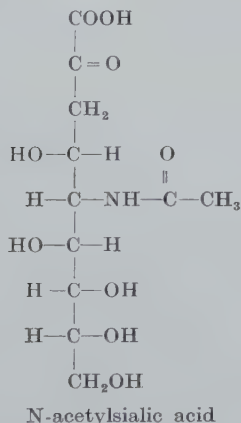
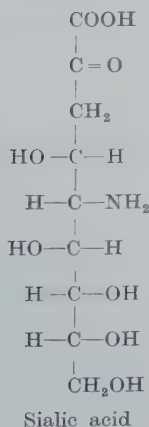
I. A colorimetric method with orcinol-hydrochloric acid (Bial's) reagent

By LARS SVENNERHOLM

With 10 figures in the text

Sialic acid is a naturally occurring carbohydrate which is found, together with hexose and hexosamine, in many proteins and derived proteins from mammals (see Winzler (1)). The amount of sialic acid often parallels that of the other two carbohydrate components. Hitherto, no pure polysaccharide has been prepared containing sialic acid. Instead, the acid seems to be an integral part of carbohydrate moieties, firmly attached to proteins or peptides (2). Furthermore, sialic acid is a component of the polysaccharide part of the gangliosides (3, 4).

More than 50 years ago the first derivative of a sialic acid was isolated (5). The definite structure has not yet been established, although the one proposed by Gottschalk (6) seems to be correct in principle. He believes that the parent compound is an aldol condensation product of 2-amino-2-deoxy-hexose with pyruvic acid.



The nomenclature in this field is still very confusing. The name *sialic acid* (7) was first applied to a substance isolated from beef submaxillary mucin by Blix in 1936 (8); this later proved to contain two acetyl groups (9). As Blix and associates (9) isolated closely related compounds from other materials, the name sialic acid was used as a group name. In my opinion it is more convenient to use the

Table 1. List of synonyms.

Comparison between the names and structure formulae in this paper and those used by other investigators.

Sialic acid ($C_9H_{17}NO_8$)	<i>Neuraminic acid</i> , Klenk <i>et al.</i> (3) ($C_{10}H_{19}NO_9$) <i>Prehemataminic acid</i> , Yamakawa and Suzuki (17, 18) ($C_9H_{17}NO_8$)
Methyl glycoside of sialic acid ($C_{10}H_{19}NO_8$)	<i>Methoxyneuraminic acid</i> , Klenk <i>et al.</i> (3, 13, 34) ($C_{11}H_{21}NO_9$) <i>Hemataminic acid</i> , Yamakawa and Suzuki (17, 18) ($C_{10}H_{19}NO_8$)
N-acetylsialic acid ($C_{11}H_{19}NO_9$)	<i>Ovine sialic acid</i> , Blix <i>et al.</i> (12) ($C_{11}H_{19}NO_9$) <i>N-acetylneuraminic acid</i> , Klenk <i>et al.</i> (34) ($C_{12}H_{21}NO_{10}$) <i>Gynaminic acid</i> , Zilliken <i>et al.</i> (14) ($C_{12}H_{21}NO_{10}$)
Methyl ester of N-acetylsialic acid ($C_{12}H_{21}NO_9$)	<i>Paramucosin</i> , Leathes (5) ($C_{12}H_{23}NO_{10}$) <i>Methoxylactaminic acid</i> , Kuhn <i>et al.</i> (15, 16) ($C_{11}H_{19}NO_9$) <i>Serolactaminic acid</i> , Yamakawa and Suzuki (19) ($C_{11}H_{19}NO_9 - CH_3OH$)
N-glycolysialic acid ($C_{11}H_{19}NO_{10}$)	<i>Porcine sialic acid</i> , Blix <i>et al.</i> (12) ($C_{11}H_{19}NO_{10}$)
N,O-diacetylsialic acid ($C_{13}H_{21}NO_{10}$, H_2O)	<i>Bovine sialic acid</i> , Blix <i>et al.</i> (12) ($C_{13}H_{21}NO_{10}$, H_2O)

name sialic acid for the parent compound as proposed in a recent paper (10). Klenk (3, 11) in 1942 proposed the name *neuraminic acid* for the parent compound. The main reasons, however, for my preferring the name of sialic acid are that Blix (8) isolated the first naturally occurring substance in this group, and later together with his associates (2, 9, 12) reported important data for the elucidation of the structure. Furthermore, Klenk and associates (13) in a recent communication do not accept the proposed structure of the basic substance and still consider the formula $C_{12}H_{21}NO_{10}$ to be valid for N-acetylneuraminic acid. On the other hand, the analytical data of the preparation used by me are clearly in favour of $C_{11}H_{19}NO_9$.

The sialic acid isolated from most materials contains an N-acetyl group, and accordingly should be designated *N-acetylsialic acid* (10). Synonyms for the same compound are *N-acetylneuraminic acid* (13) and *gynaminic acid* (14). The methyl ester of N-acetylsialic acid has been named *methoxylactaminic acid* by Kuhn and Brossmer (15, 16). For further details see Table 1.

Several methods for the determination of sialic acid have been developed: Bial's reaction (20, 21), direct Ehrlich reaction (20), the diphenylamine reaction (22, 23), the tryptophane-perchloric acid reaction (24), and the hydrochloric acid method of Folch (25). Moreover, orcinol in Bial's reagent has been replaced by resorcinol (26).

However, the value of these quantitative estimations is restricted, as it is often impossible to find out to which substance the reported data refer. Blix and collaborators (20, 27–29) have apparently calculated their values as *N,O*-diacetyl sialic acid. Klenk and associates (21, 30, 31), and Böhm and associates (32, 33) have used *methoxyneuraminic acid* as a reference substance, but whether the values are calculated as methoxy neuraminic acid or as only neuraminic acid is not evident.

In the present study it has been demonstrated that the molar absorbancy indices of *N*-acetylsialic acid and *N,O*-diacetylsialic acid are the same. *N*-acetylsialic acid can be isolated in a high yield in pure form (14) and is stable for months in dilute aqueous solution at 4°C. Therefore, it is very suitable as a reference substance. Klenk, on the other hand, has found *N*-acetylneuraminic acid to be stable only for a few days. It seems reasonable to assume that his preparations contained small amounts of mineral acids, which rapidly reduce the stability of *N*-acetyl sialic acid. *N,O*-diacetylsialic acid is not suitable as a reference substance, as a uniform preparation has not yet been obtained (12). The isolation of pure methoxyneuraminic acid appears to be simple. Further investigations are, however, demanded on the relationship of the molar absorbancy indices between *methoxyneuraminic acid* and *N*-acetylsialic acid before methoxyneuraminic acid can be accepted as an alternative reference substance.

Already in 1885, Cyrill Reichl (35) (quoted by Reinitzer (36)) introduced orcinol in hydrochloric acid as a reagent for the estimation of pentoses. Later on Salkowski (37) used the same reagent for the detection of pathological quantities of pentoses in urine. Manfred Bial (38) in 1902 tried the reagent for the same purpose, but he found that the method was not sensitive enough for clinical use. By adding an oxidative agent as FeCl_3 , CuSO_4 or HgO the sensitivity was increased several times. Only minor modifications of the reagent have been done by other research workers (39–42), when applying the method for the quantitative estimation of pentoses.

In 1903, Levene (43) in a study of the nucleic acids of the spleen, obtained a by-product which was rich in carbohydrate, contained sulphuric acid and gave a violet colour when boiled with orcinol and hydrochloric acid. The material was referred to as "glycothionic acid", an early name for the sulphuric acid-containing carbohydrates of the body. Similar materials giving the same colour reaction and containing sulphur were later on isolated by Mandel and Levene (44) from *inter alia* mammary gland, liver and kidney. These "glycothionic acids" were not obtained in a pure state and not further studied.

About 20 years later Levene together with Landsteiner (45) on investigating the heterogenetic hapten isolated from the protagon fraction of horse kidney a water-soluble sugar-containing lipid. This, contrary to the cerebrosides, also gave a strong purple colour with orcinol, hydrochloric acid and copper sulphate or ferrous sulphate. Independently of Landsteiner and Levene, Walz (46) isolated a glycolipid from spleen and brain, which also gave the same colour with Bial's reagent. Klenk (47, 48) also used Bial's method when he demonstrated that the brain in Niemann–Pick's and Tay–Sachs' diseases contained increased amounts of the new glycolipid. Later on, Klenk (3) studied the new lipid more closely and gave it the name of ganglioside. Blix (49) applied the reagent as a quantitative tool to follow the isolation of the lipid from normal brains.

In 1941, Klenk and Langerbeins (21) worked out a quantitative application of Bial's method for the estimation of sialic acid (neuraminic acid) in nervous tissue. The latter method was tried by Brante and Svennerholm (50) for the determination

of gangliosides in different types of nervous material. We found that the method could not be applied to normal brain tissue without correction for contaminating substances. Odin and Werner (20) modified the procedure of Blix (49) and determined the absorbancy indices of several naturally occurring carbohydrates as well. Böhm, Dauber and Baumeister (32) performed the Klenk-Langerbeins (21) method at a lower temperature. They also estimated the absorbancy indices of some carbohydrates normally occurring together with sialic acid in proteins.

On account of the great lability of sialic acid in alkaline and acid media, estimations of the acid in tissues and other materials based on the isolation of the acid may always result in too low values. Colorimetric methods applied direct to the material are therefore of a considerable value for the quantitative determination of sialic acid. Before using a method the factors affecting the colour formation must be thoroughly examined. Only Klenk and Langerbeins (21) have performed such an investigation. The authors referred to above (20, 32) have determined the percentage absorbancies of the contaminating substances and have corrected the absorbancy readings. However, they have not checked whether the contaminants follow Beer's law, and whether the absorbancies are additive in a mixture of sialic acids and carbohydrates. Werner and Odin (20) have also made an error when calculating the absorbancy coefficients of the hexoses from the absorption curves, so that these values are ten times too high.

It may therefore be appropriate to describe the factors influencing the colour formation of sialic acid and other carbohydrates with Bial's reagent.

Experimental

Materials

Sialic acids

N,O-diacetylsialic acid ($C_{13}H_{21}NO_{10}$, (H_2O)) from beef submaxillary mucin and N-glycolylsialic acid ($C_{11}H_{19}NO_{10}$) from pig submaxillary mucin were put at my disposal by Professor G. Blix, Uppsala. The purity of these substances is discussed by Blix *et al.* (12) in a recent paper. Several different samples of N-acetylsialic acid ($C_{11}H_{19}NO_9$) were prepared from human serum, milk, gangliosides and brain proteins by using the method described (10). In the colorimetric tests used no preparation showed larger deviation than -5 % from the greatest value. The acid with the maximum absorbancy index was used in this investigation. N, 4.55; CH_3CO , 13.90. Molecular weight 308 (calc. 309.3) (potentiometric titration). The acid gave one single spot at paper chromatography in four different solvents (14).

Other carbohydrates

Materials (whenever possible analytical reagents or C.P. grade reagents) were obtained from the following sources; D-glucose, D-mannose, and L-rhamnose from British Drug House Ltd.; D-galactose, D-fructose and L-sorbose from E. Merck; L-fucose, D-ribose, D-xylose and D-arabinose from Pfanstiehl Chemical Co.; glucuronic acid lactone from Sigma Chemical Co., and digitoxose from Delta Chemical Works, Inc. The carbohydrates were all recrystallized at least once and dried over P_2O_5 and NaOH pellets to constant weight in a vacuum desiccator. Ribulose was prepared from D-arabinose acc. to Glatthaar and Reichstein (50). D-glucoheptulose and 2,7-anhydro- β -D-altro-heptulo-pyranose monohydrate, prepared by Professor N. K.

Richtmeyer, National Institutes of Health, Bethesda, were a gift to Professor Nordal, Department of Pharmacognosy, Oslo, and placed at my disposal by him. As a source of deoxyribose, thymonucleic acid (Nutritional Biochemicals Co.) was used. D-glucosamine-HCl (N, 6.45; $(\alpha)_D^{20} = +72^\circ$) was prepared from umbilical cord by the author. D-galactosamine-HCl was a gift from Docent S. Gardell, Chemistry Department II, Karolinska Institutet, Stockholm. Furfural A. R. (E. Merck) was freshly redistilled *in vacuo* before use.

Orcinol (Merck) was recrystallized from benzene and dried to constant weight in a vacuum desiccator over P_2O_5 . For routine work no recrystallization is necessary.

Hydrochloric acid (Merck) A. R., density 1.19, at least 36.4 % HCl. Fe^{3+} not more than 0.0001 %.

0.1 M-Fe³⁺. 48.22 g of $NH_4Fe(SO_4)_2$, 12 H_2O was dissolved in distilled water, 50 ml concentrated hydrochloric acid was added and afterwards distilled water to 1 l.

0.1 M-Cu²⁺. 24.97 g of $CuSO_4$, 5 H_2O was dissolved in distilled water to 1 l.

Amyl alcohol. 1 l of isoamyl alcohol (purum, May and Baker) was mixed with 200 ml concentrated hydrochloric acid and left for a week. The alcohol was washed with 200 ml water about ten times, dried with anhydrous K_2CO_3 and distilled. The fraction with b.p. 130–133° (uncorrected) was used.

Standard method

Orcinol reagent. 0.2 g orcinol was dissolved in 10 ml of distilled water, 80 ml of concentrated hydrochloric acid A.R. and 1.00 ml of 0.1 M- Fe^{3+} or 0.25 ml of 0.1 M- Cu^{2+} was added. The volume was made up to 100 ml with distilled water. The reagent was prepared at least 4 hours before use. It was stable at least for one week when stored in the refrigerator at 0°C.

Blank reagent. The same as above but without orcinol.

Procedure. Samples of 2 ml, containing 10–40 μg of N-acetylsialic acid or corresponding amount of other sialic acids, were pipetted into three tubes (18 × 200 mm) with glass stoppers (Thunberg tubes). 2 ml of orcinol reagent were added to two of the tubes (test samples), and 2 ml of blank reagent to the third tube (sample blank). The tubes were heated at 106°C for 15 minutes in an oil bath or in a boiling water bath and afterwards chilled in running water to room temperature. 5 ml of isoamyl alcohol were added and the tubes vigorously shaken. The rack with the tubes was then placed in ice water for 10 minutes. The contents of the tubes were transferred to centrifuge tubes and the tubes spun at 500–1000 r.p.m. for one minute. After centrifugation the tubes were placed in the ice water again and left there until the photometric reading was done. The amyl alcohol phases were transferred to 50 mm microcells with ballooned pipettes and read at 575 and 680 $m\mu$ in a Beckman spectrophotometer Model B against pure amyl alcohol.¹ The readings were completed within one hour after heating.

Calculation. The absorbancy of the blank samples was subtracted from that of the test samples and the amount of N-acetylsialic acid calculated by means of the following equation:

$$\text{N-acetylsialic acid} = \frac{(A_s)_{575} - (A_s)_{680}}{(mg/5 \text{ ml})} \cdot \frac{(a_s)_{575} - (a_s)_{680}}$$

¹ The sample holder for microcells was manufactured by Mr. H. Tillquist, Stockholm, designed by K. Agner, M.D.

Absorptiometric terms used

The terms and symbols used in absorptiometric and colorimetric work are very confusing in their definitions, usages and conventions. The recommendations given in Letter Circular LC-857 of the National Bureau of Standards have been used as the general basis in *Analytical Absorption Spectroscopy* (51). The nomenclature proposed in the book has been used in this paper.

T_s = transmittancy, the ratio of the transmittance of the solution to that of the solvent in equal thickness.

A_s = absorbancy.

b = cell thickness in centimetres.

c = concentration in units of grams per 1000 cc.

$a_s = A_s/bc$ = absorbancy index in units of 1000 sq.cm per gram.

$a_M = 1000 A_s/bM$ = molar absorbancy index, c being expressed in moles per litre.

Experimental results

The colour formation of sialic acids and other carbohydrates with orcinol and hydrochloric acid depends on several factors and varies considerably with the experimental conditions. The influence of these factors was investigated in order to obtain optimum conditions for the reaction. The colour depends mainly on the presence of Fe^{3+} or Cu^{2+} in the reagent. The intensity of the colour also depends on the concentration of hydrochloric acid and orcinol and on the temperature and time of heating. The standard procedure was followed and, as a rule, only one factor was changed in each experiment. The experiments were done both with orcinol-hydrochloric acid reagent containing Fe^{3+} (Fe-Bial) and Cu^{2+} (Cu-Bial).

Hydrochloric acid concentration

The effect of varying the hydrochloric acid concentration was studied with N-acetylsialic acid and galactose (Fig. 1). The absorbancy augmented with increasing hydrochloric acid concentration up to a final acid concentration of 5.75 *N*. In the standard procedure the final acid concentration is only 4.8 *N*, but the difference in molar absorbancy is not more than 5%. The absorbancy of galactose increased more rapidly with increasing acid concentration. Thus, there is no reason to change the original acidity of the reagent. The position of the absorption maximum of N-acetylsialic acid was not influenced by the variance of the hydrochloric acid concentration.

Orcinol concentration

The effect of varying the orcinol concentration was studied with N-acetylsialic acid. Up to 100 mg orcinol/100 ml reagent the absorbancy increased rapidly (Fig. 2). With higher concentrations of orcinol, a minor increase of the absorbancy occurred. With higher concentrations of Cu^{2+} or Fe^{3+} than in the standard method the maximum absorbancy was reached at a higher level of orcinol (the same observation has been made by Jarrige (53) at the determination of glucuronic acid). The disadvantage of using greater concentrations of these ions is discussed under the following section. The original reagent contains 200 mg orcinol/100 ml reagent, which is a suitable concentration.

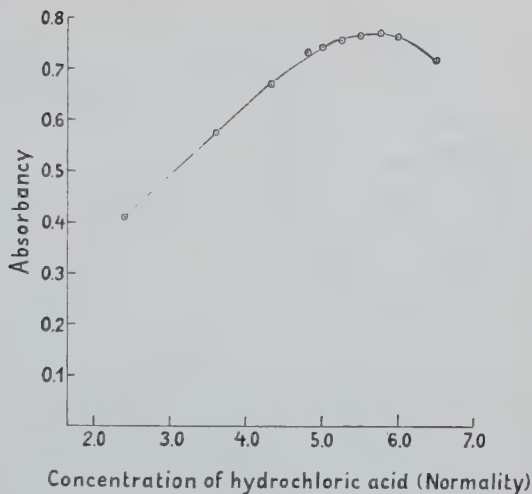


Fig. 1. Change in absorbancy with concentration of hydrochloric acid. The absorbancy at $575\text{ m}\mu$ was read against a reagent blank with the same strength of the acid. $40\text{ }\mu\text{g}$ N-acetylsialic acid. Fe-Bial.

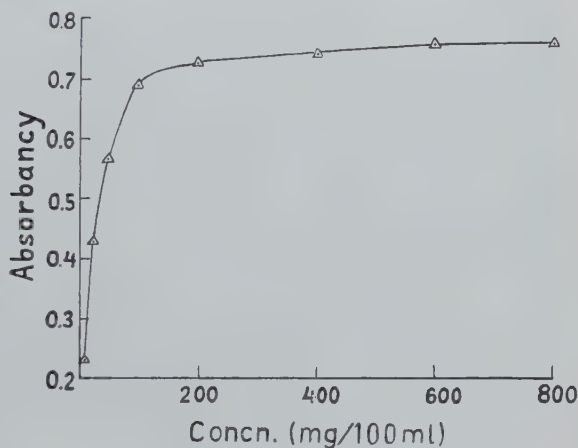


Fig. 2. Change in absorbancy with concentration of oreinol in the reagent. The absorbancy at $575\text{ m}\mu$ was read against a reagent blank with the same concentration of oreinol. $40\text{ }\mu\text{g}$ N-acetylsialic acid. Fe-Bial.

Metal ions

A certain amount of oxidizing agent in Bial's reagent is necessary for maximum colour formation. In the reagent used for quantitative estimation of sialic acid (neuraminic acid) only ferric chloride has been used earlier. As it is difficult to weigh out an exact amount of ferric chloride and the amount of Fe^{3+} is of great importance for the colour development of both sialic acids and contaminating sugars, ferric ammonium sulphate was used as a source of Fe^{3+} . As can be seen in Fig. 3, optimal colour formation was achieved with about $1\text{ mM-Fe}^{3+}/1000\text{ ml}$ reagent when the concentration of N-acetylsialic acid varied between $10\text{--}60\text{ }\mu\text{g}$. A decrease in the concentration of Fe^{3+} was followed by a diminished colour formation. To test whether no colour at all was formed, if all metal ions were removed as completely as possible, the water used was distilled twice (apparatus of boro silicate glass), and the hydrochloric acid solution was prepared by leading purified hydrogen chloride into the distilled water. All glass

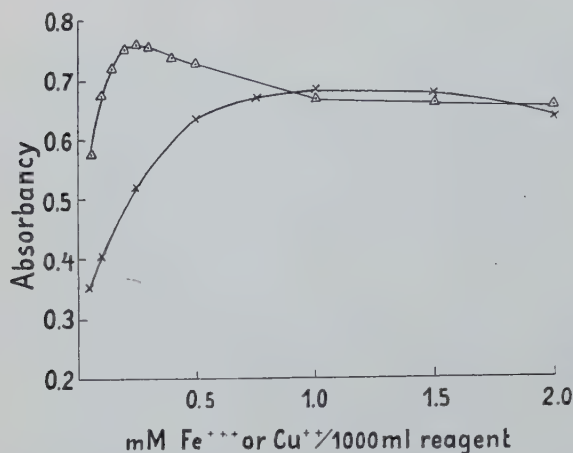


Fig. 3. Change in absorbancy with concentration of Δ , Cu^{2+} and $\times$, Fe^{3+} in the reagent. The absorbancy was read at $575\text{ m}\mu$ against a reagent blank with the same concentration of Fe^{3+} or Cu^{2+} ; $38\text{ }\mu\text{g}$ N-acetylsialic acid.

were used was cleaned in the prepared hydrochloric acid and redistilled water. Also after these precautions the absorbancy was about $\frac{1}{4}$ of the maximum one (Table 4). The effect of other cations on the absorbancy index of N-acetylsialic acid was also tested. As can be seen in Table 2, the absorbancy was increased by Cu^{2+} and to a lesser extent by Ag^+ . Contrary to Bial's findings no effect was obtained with Hg^{2+} .

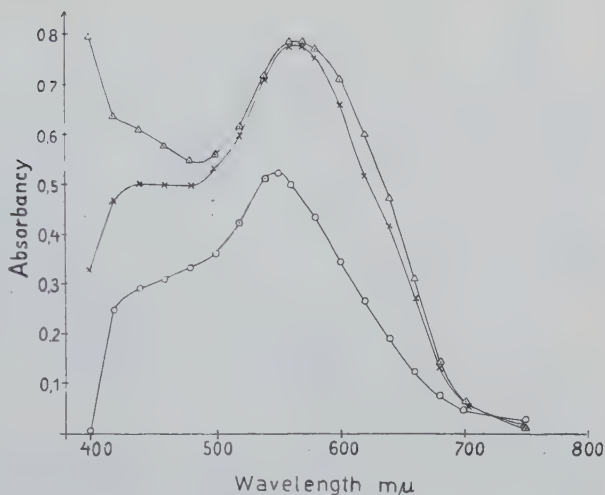
In the standard procedure the absorption maximum of sialic acid is situated at $575\text{ m}\mu$, but with decreasing concentration of Fe^{3+} the maximum was changed to a lower wavelength (Fig. 4). When no Fe^{3+} was added the absorption maximum was at $545\text{ m}\mu$, while addition of more than $1\text{ mM-Fe}^{3+}/1000\text{ ml}$ reagent did not change the position of the maximum.

Table 2. The effect of different cations on the absorbancy of N-acetylsialic acid in orcinol-hydrochloric acid reagent.

1 ml of a 0.1 M solution of the cation was added to 100 ml reagent. $26\text{ }\mu\text{g}$ N-acetylsialic acid. Standard procedure.

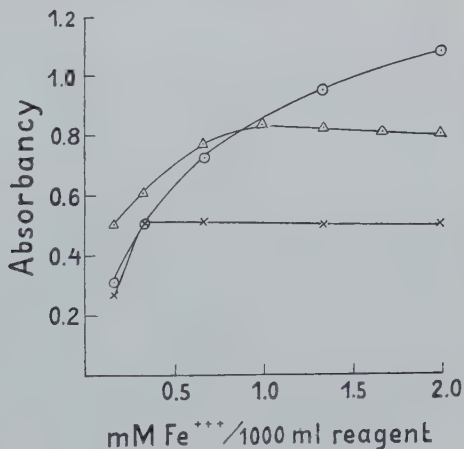
Cation added to orcinol-hydrochloric acid	Absorbancy at $575\text{ m}\mu$
None	0.115
Ag^+	0.220
Ba^{2+}	0.105
Bi^{3+}	0.136
Ca^{2+}	0.111
Cd^{2+}	0.120
Co^{3+}	0.133
Cu^{2+}	0.488
Fe^{3+}	0.508
Hg^{2+}	0.110
Mg^{2+}	0.119
Mn^{2+}	0.108
Na^+	0.114
Ni^{2+}	0.119
Zn^{2+}	0.123

Fig. 4. Absorbancy-wavelength curves for N-acetylsialic acid at different concentrations of Fe^{3+} in the reagent. $\circ$, Fe^{3+} not added, 110 μg N-acetylsialic acid; $\times$, 0.5 mM- Fe^{3+} , 44 μg N-acetylsialic acid; $\triangle$, 2.0 mM- Fe^{3+} , 44 μg N-acetylsialic acid. The samples were read against a reagent blank containing 0.5 mM- Fe^{3+} .



The absorbancy indices of other carbohydrates also depend on the concentration of Fe^{3+} . With the amounts of pentoses tested (10–100 μg) the optimal concentration of Fe^{3+} was the same as for the sialic acids. The hexoses—galactose, glucose and mannose—tested at the same absorbancy level as that of sialic acids and pentoses, require a larger Fe^{3+} concentration for maximum colour yield. The amount of hexoses tested was 20–40 times higher than that of sialic acids. When 10-fold amounts of sialic acids and pentoses were tested a larger concentration of Fe^{3+} in the reagent was necessary for maximum colour formation. It is thus evident that the absorbancy is a function of the formation of “sugar”-orcinol- Fe^{3+} (Cu^{2+}) complex. Although it seems reasonable to work with a large excess of orcinol and Fe^{3+} or Cu^{2+} , several disadvantages were found: The yellow-green reagent colour was strong enough to hide the red-violet colour of sialic acid. Beer’s law was valid within a shorter range owing to destruction of sialic acid by humin formation or precipitation of formed

Fig. 5. Change in absorbancy with concentration of Fe^{3+} in the reagent. $\triangle$, 44 μg N-acetylsialic acid; $\times$, 50 μg ribose; $\circ$, 2.0 mg galactose. The absorbancy was read at 575 mμ against a reagent blank with the same concentration of Fe^{3+} .



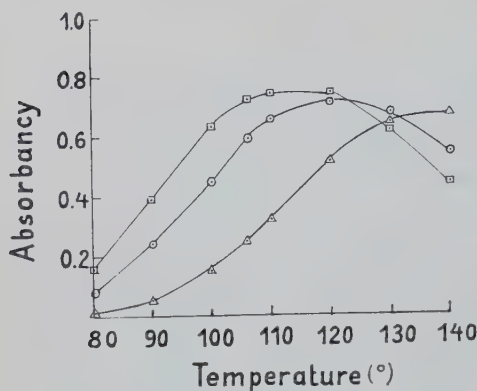


Fig. 6. Change in absorbancy with temperature and time of heating. 40 μ g of N-acetylsialic acid was heated for Δ , 5 min.; $\odot$, 10 min.; $\square$, 15 min.; at different temperature. The absorbancy was read at 575 $m\mu$ against a reagent blank heated at the same conditions. Fe-Bial.

complex. The amyl alcohol phase was inhomogenous and a strong coloured ring was often layered upon the water phase. Duplicate determinations often showed large deviations. Thus, a good reproducibility was only achieved when the analyses were done with the low concentrations of sialic acid and orcinol- Fe^{3+} (Cu^{2+}) which were used in the standard method.

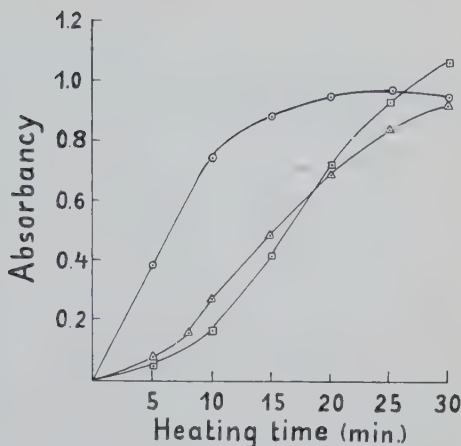
When Levene and Landsteiner (45) identified the lipids of the protagon fraction, they used an orcinol reagent containing copper sulphate instead of ferric chloride. According to my knowledge nobody has afterwards used copper sulphate in the reagent for the determination of sialic acid. This is remarkable, as an exact amount of copper sulphate, but not ferric chloride, can be weighed out. In the experiments performed with Cu^{2+} -containing reagent no disadvantage was found. The maximum molar absorbancy index achieved with Cu-Bial was about 5 % higher than that with Fe-Bial (Fig. 3). The amount of Cu^{2+} necessary for maximum absorbancy was only $\frac{1}{4}$ of that of Fe^{3+} . The colour of the reagent was much weaker which made it possible to visualize the red-violet colour of sialic acid in lower concentrations than with Fe-Bial.

Time of heating and temperature

As might be expected, the time of heating necessary to obtain maximum absorbancy depends on the temperature used (Fig. 6). The highest molar absorbancy was achieved with a lower temperature for a longer time. The greatest advantage with an increased time of heating is that a small difference in time will cause only minor differences in the absorbancy values. A very constant molar absorbancy index of N-acetylsialic acid has been found after heating for 15 minutes at 106° which contrasts with the considerably larger deviations found with the method of Klenk and Langerbeins in which the samples are heated only 5 minutes at 142°. At the standard conditions the maximum absorbancy was not achieved but if the time of heating was prolonged the influence of contaminating substances was increased much more (Fig. 7).

The experiments described were performed with pure samples of sialic acids. When crude preparations of gangliosides and serum proteins were tested the development of colour was delayed (Table 3). In materials with a low content of sialic acid the amounts found are consequently lower if the time of heating is short. Klenk and Faillard (31) also found the content of sialic acid in submaxillary mucin to increase after hydrolysis.

Fig. 7. Absorbancy-heating time curves of $\circ$, 50 μ g N-acetylsialic acid; $\triangle$, 50 μ g ribose; $\square$, 1.0 mg galactose. The absorbancy was read at 575 $m\mu$ against a reagent blank heated under the same conditions. Fe-Bial.



Stability of the colour

A great disadvantage in the method is the instability of the formed colour. As can be seen from Table 4, the absorbancy decreased rapidly, if the tubes were left at room temperature. The decrease was lower, if the tubes were cooled rapidly, centrifuged and then placed in ice water until the reading was done. Dische and Schwarz (39) made the same observation at the estimation of pentoses.

Behaviour of different sialic acids

Blix and associates (9) have isolated three well-characterized sialic acids with different compositions: N-acetylsialic acid, N,O-diacetylsialic acid and N-glycolylsialic acid. It was of great importance to investigate the absorption curves and molar absorbancy indices of the substances in the orcinol hydrochloric acid method.

The absorbancy indices were not identical. As seen from Tables 5 and 6, N-acetylsialic acid and N,O-diacetylsialic acid have indices of equal magnitude,¹ if the origin-

Table 3. Rate of colour development.

The samples were treated according to the standard procedure. The absorbancies were corrected by dichromatic readings. Mean values of four estimations.

Material	Amount mg	Absorbancy			Per cent colour development	
		Time of heating at 106°				
		5 min.	10 min.	15 min.	5 min.	10 min.
N-acetyl sialic acid	0.040	0.164	0.535	0.611	26.8	87.6
Gangliosides	0.22	0.178	0.724	0.847	21.0	85.5
Serum proteins	1.4	0.134	0.610	0.745	18.0	81.4

¹ The O-acetyl group in N,O-diacetylsialic acid is easily split off so that shortly after adding the reagent the samples contain only N-acetylsialic acid.

Table 4. Change of absorbancy with time.

20 samples with the same amount of N-acetylsialic acid were treated in the general way. The absorbancy of 4 samples was read as soon as possible after finished heating (15 min.). Of the remaining 16 samples, 8 samples were left at room temperature, and the other 8 placed in ice water. Two of each were read at intervals of 15 min.

Minutes after finished heating	Standing in air temp. 20°C	Standing in ice water
	Absorbancy at 575 m μ	
15	0.652	
30	0.630	0.651
45	0.627	0.649
60	0.623	0.641
75	0.606	0.625

Table 5. Estimation of the molar absorbancy indices of sialic acids and other carbohydrates. Fe-Bial.

Standard method. The samples were read against a reagent blank. The readings were done at 575 m μ and at the absorption maxima of the substances tested.

Material	Amount (mg)	A_s	$(a_s)_{575\text{ m}\mu}$	$(A_M)_{575\text{ m}\mu}$	$(A_M)_{\max_1}$	$(A_M)_{\max_2}$
N-acetylsialic acid $C_{11}H_{19}NO_9$	0.0417	0.756	18.14	5605		
N,O-diacetylsialic acid $C_{13}H_{21}NO_{10}, H_2O$	0.046	0.686	14.92	5505		
$C_{13}H_{21}NO_{10}$				5237		
N-glycolylsialic acid $C_{11}H_{19}NO_{10}$	0.03936	0.817	20.75	6744		
Galactose	1.00	0.566	0.566	102	570 m μ	670 m μ
					104	103
Glucose	1.00	0.575	0.575	104	560 m μ	670 m μ
					111	73
Mannose	1.00	0.870	0.870	157	560 m μ	670 m μ
					163	108
Arabinose	0.05	0.192	3.84	576		675 m μ
						1770
Ribose	0.05	0.470	9.40	1410		675 m μ
						3880
Glucuronic acid lactone .	0.10	0.455	4.55	883		675 m μ
						2053
Deoxyribose	0.21	0.606	2.89	387		670 m μ
						603
Fructose	0.05	0.632	12.64	2275	555 m μ	670 m μ
					2635	835
Sorbose	0.05	0.757	15.14	2725	560 m μ	
					2916	
Fucose	1.00	0.450	0.450	74	605 m μ	660 m μ
					80	96
Rhamnose	1.00	0.626	0.626	114	555 m μ	660 m μ
					118	108

Table 6. Estimation of the molar absorbandy indices of sialic acids and other carbohydrates. Cu-Bial.

Standard method. The samples were read against a reagent blank. The readings were done at 575 $m\mu$ and at the absorption maxima of the substances tested.

Material	Amount (mg)	A_s	$(a_s)_{575\ m\mu}$	$(A_M)_{575\ m\mu}$	$(A_M)_{\max_1}$	$(A_M)_{\max_2}$
N-acetylsialic acid $C_{11}H_{19}NO_9$	0.0417	0.795	19.06	5895		
N,O-diacetylsialic acid $C_{13}H_{21}NO_{10}, H_2O$	0.046	0.722	15.70	5798		
$C_{13}H_{21}NO_{10}$				5515		
N-glycolylsialic acid $C_{11}H_{19}NO_{10}$	0.03936	0.854	21.70	7060		
Galactose	1.00	0.471	0.471	85	565 $m\mu$ 86	670 $m\mu$ 90
Glucose	1.00	0.539	0.539	97	560 $m\mu$ 104	670 $m\mu$ 67
Mannose	1.00	0.728	0.728	131	560 $m\mu$ 136	670 $m\mu$ 92
Ribose	0.025	0.259	10.36	1554		670 $m\mu$ 3960
Glucuronic acid lactone .	0.10	0.336	3.36	585		670 $m\mu$ 1566
Furfural	0.0232	1.015	43.75	4200	570 $m\mu$ 4241	670 $m\mu$ 5110
Fructose	0.10	0.890	8.9	1602	555 $m\mu$ 1800	670 $m\mu$ 936
Sorbose	0.05	0.599	11.98	2156	560 $m\mu$ 2360	
Glucoheptulose	0.01132	0.380	33.57	7050		640 $m\mu$ 13360
2,7-anhydro- β -d althroep- tulose	0.01846	0.800	43.34	9101		640 $m\mu$ 17520
Fucose	1.00	0.322	0.322	53		660 $m\mu$ 80
Rhamnose	1.00	0.629	0.629	115	600 $m\mu$ 123	660 $m\mu$ 140
Galactosamine-HCl . . .	1.00	0.024	0.024	5.2		
Glucosamine-HCl	1.00	0.019	0.019	4.1		

ally proposed formula for diacetylsialic acid ($C_{13}H_{21}NO_{10}, H_2O$) is used for the calculations. In their last paper, Blix and associates (12) considered the formula $C_{13}H_{21}NO_{10}$ more probable. If this formula is used the molar absorbandy index will be about 5 % lower. As the substance is not homogeneous (12) and the preparation used for the test contained small amounts of hexose, the agreement is satisfactory with both the proposed formulae. N-glycolylsialic acid on the contrary has a higher molar absorbandy index than the other two, 120 % of that of N-acetylsialic acid. The same difference was also found with the other methods used for the determination of sialic acids (26). Thus, in materials only containing N-glycolylsialic acid the estimated figure will be about 20 % too high when N-acetylsialic acid is used as standard substance. Fortunately, N-glycolylsialic acid has only been detected in pig submaxillary mucin.

Under the conditions used, Beer's law was valid for all the three sialic acids in-

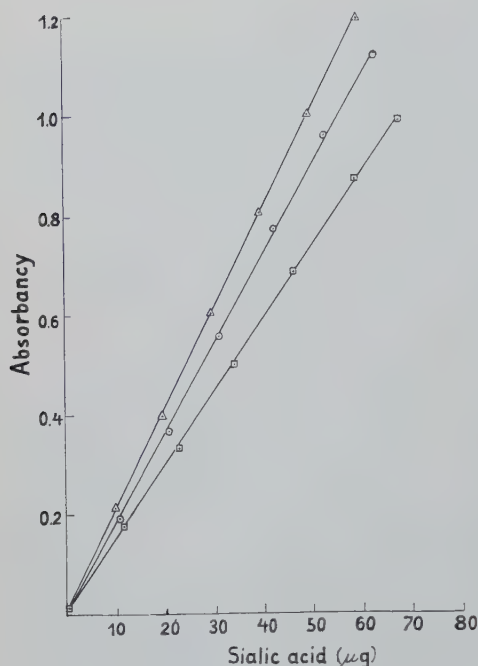


Fig. 8. Absorbance-concentration curves of $\square$, N,O-diacetylsialic acid; $\circ$, N-acetylsialic acid; $\triangle$, N-glycolylsialic acid. The absorbance was read at 575 $m\mu$ against a reagent blank. Cu-Bial.

vestigated (Fig. 7) up to an amount of 60–75 μg in the samples. When analysing larger amounts than 75 μg of sialic acids a deviation from the theoretical curve occurred which was 5–8 % with amounts of 250 μg . If still larger amounts were tested, the absorbance at 575 $m\mu$ decreased rapidly, owing to destruction of the sialic acids by humin formation. The slight deviation, observed with moderately increased amounts of sialic acids, was partly due to insufficient concentration of Fe^{3+} in the reagent. A further addition of Fe^{3+} to the reagent decreased the deviation, but the theoretical curve was not reached, respectively increased the molar absorbance indices of the hexoses (Fig. 4).

Behaviour of other carbohydrates

As previously mentioned, Bial's reagent has mainly been used for the quantitative estimation of pentoses. These develop a bluegreen colour with an absorption maximum at 670 $m\mu$. The absorption curve-shapes of the different aldopentoses were identical, but the molar absorbance indices were quite different, if the heating time was not prolonged to about half an hour, which has also been demonstrated by other investigators (40, 41, 54). In addition to an absorption maximum at 670 $m\mu$, ketopentoses (55, 56) have a second one at about 540 $m\mu$. The absorption curve shapes of glucuronic acid was also the same as that of aldopentoses. Furfural had a maximum at 670 $m\mu$ but, also, a second one at 570 $m\mu$. It has been suggested that the colouring matter formed from pentoses with Bial's reagent is a condensation product of furfural

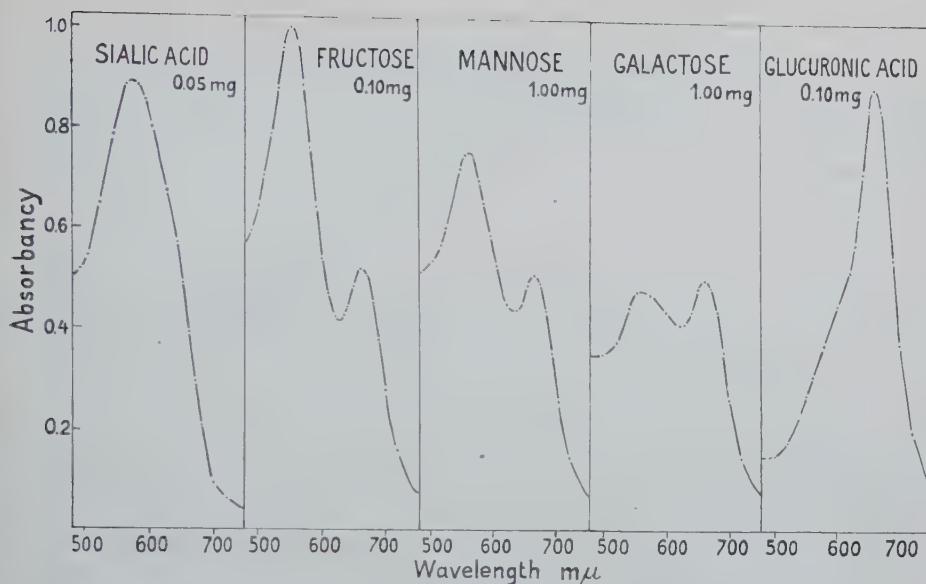


Fig. 9. Absorbance-wavelength curves of N-acetylsialic acid and some other carbohydrates. Cu-Bial.

and orcinol. The difference in absorption curve shapes of pentoses and furfural is so pronounced that the mechanism must be more complex. Adenosine-5-phosphate under certain conditions was also shown to give a higher absorbance with Bial's reagent than free pentoses though the yield of furfural was lower when heated with acid (39, 40).

The hexoses are also known to give coloured products with orcinol but to a much lesser extent. The suggestion that the colour depends only on the conjugation of formed hydroxymethylfurfural to orcinol must be erroneous. The aldohexoses tested had two absorption maxima, one at 550–560 $m\mu$ and the second at 670 $m\mu$, but the absorption spectra of galactose and mannose, for instance, were quite different (Fig. 9). Apart from furfural and its homologues a large number of intermediary products related to, or derived from, furfural are produced by the action of strong acids on sugars (57). This is illustrated by the behaviour of the ketohexoses. When less than 0.1 mg of fructose and sorbose was tested a red violet colour appeared, impossible to differentiate from that of sialic acid without a spectrophotometer (Fig. 9). When on the other hand amounts larger than 0.1 mg of fructose were used a colour was formed, which could not be differentiated from that of aldohexoses. As can be seen in Fig. 10, in which the absorbances at 555 $m\mu$ and 670 $m\mu$ are given for different concentrations of fructose, Beer's law is only valid within a narrow concentration range. The ratio of the absorbance at 555 $m\mu$ to that at 670 $m\mu$ diminishes rapidly with increasing concentrations of fructose when the redviolet colour of the sample changes to yellow-green. The redviolet colour given by fructose in low concentration has not been observed by early investigators which is explained by the fact that high concentrations were used for testing. A "positive" colour test with

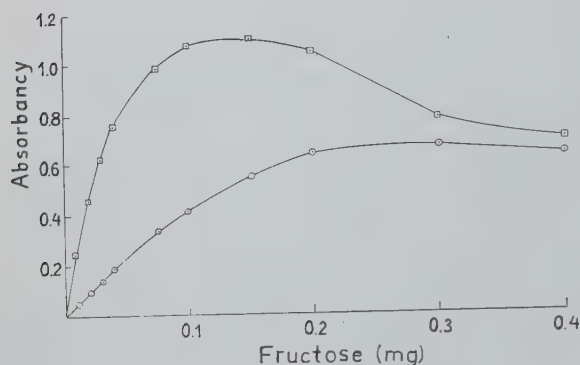


Fig. 10. Absorbance-concentration curves of fructose. □, at 555 mμ; ○ at 670 mμ. Cu-Bial.

Bial's reagent is thus no proof of the existence of sialic acids, when ketohexoses also occur in the sample.

The sialic acids and the ketohexoses also behaved similarly when larger amounts were tested. Already after boiling the samples for 5–10 minutes a voluminous brown-black precipitate was formed. The difference between sialic acids and ketohexoses is the lack of an absorption maximum or plateau at 670 mμ of the sialic acids, owing to the blocking effect of the amino group on formation of furfural derivatives. Furthermore, Beer's law was valid within a wider concentration range for the sialic acids than for the ketohexoses.

From the data obtained it seems possible that Bial's reagent acted partly as a furfural reagent, partly as a ketose reagent. Bial's test was therefore performed on the other ketosugars available, the ketoheptoses. These sugars gave only one absorption maximum at 650 mμ but the molar absorbance indices were of the same order as those of the other keto-sugars tested; in fact they had the greatest molar absorbance indices of all substances tested. Nordal and Klevstrand (58) have earlier worked out a modified Bial's method for the estimation of ketoheptoses and they also found the absorbance coefficient of sedoheptulose to be large.

Gottschalk (59) has discussed the behaviour of sialic acid in the diphenylamine method of Dische (29, 23). Sialic acids give a colour reaction similar to 2-deoxyhexoses (with an absorption maximum at 530 mμ), and he regards the decarboxylated sialic acid as a substituted 2-deoxyhexose. It is probable that this structural feature is responsible for the colour formation with the diphenylamine reagent, but digitoxose, tested with Bial's reagent, gave the same type of absorption curve as the aldohexoses. Bial's test may instead be assumed to be a ketose reaction, and the structural feature of sialic acid responsible for the red violet colour in the test to be the α-keto group.

In Tables 5 and 6 the molar absorbance indices of the different substances are listed, partly at 575 mμ, partly at the absorption maxima. The readings were performed in a Beckman spectrophotometer model B with 50 mm microcells and a slit width of about 0.1 mm. As Beer's law was valid only up to an absorbance of about 0.600 for pentoses (tested with arabinose, ribose and xylose) and to about 0.400 for hexoses (galactose, glucose and mannose) and 6-deoxyhexoses, the amount of sugar used for the test is also given. From the calculated absorbance indices it is evident that ketosugars, pentoses, and glucuronic acid have much higher absorbance indices than the other sugars.

Behaviour of other substances than carbohydrates

Bial's test was also performed on substances other than carbohydrates normally occurring in biological materials. The following substances gave no absorbancy: the common amino acids, creatine, creatinine, choline, ethanolamine, glycerol, glycerophosphoric acid, inositol, purines, pyrimidines, urea and uric acid.

Determination of sialic acid in biological materials

As the samples also contain other carbohydrates than sialic acid it is necessary to compensate for their absorbancy in Bial's test. Werner and Odin (20) and Böhm, Dauber and Baumeister (32) determined the hexose content of the samples with an orcinol-sulphuric acid or anthrone-sulphuric acid method. From a table of the absorbancy coefficients of hexoses in Bial's test their absorbancy was calculated and subtracted from the readings of the samples. The method seems quite correct and is also applicable to samples containing small amounts of other sugars. But for other samples the procedure gave too low values. As it was found that the absorbancies were additive up to an amount of about 500 μg of hexose, the reason may be that the glycosidic bound hexoses have lower absorbancy coefficients than the free ones.

The following experiment was done to illustrate that the procedure earlier used was not suitable for the evaluation of the influence of other carbohydrates on estimated sialic acid in biological samples. Varying amounts of cerebrosides, dissolved in chloroform-methanol, were added to a pure preparation of gangliosides dissolved in the same solvent. The solvent was evaporated, and after emulsification of the lipid mixture in water, Bial's test was performed. The results are given in Table 7. As seen from the table, the deviation from the calculated values increased rapidly with greater concentrations of cerebrosides.

As an alternative, the problem can be solved by reading samples also at a second wavelength, where sialic acid and the other carbohydrates have not the same absorbancy coefficients as at the first wavelength. The second wavelength is selected so that the absorbancy coefficients of the contaminants are the same at the two wave-

Table 7. Estimation of sialic acid in samples also containing galactose.

To samples containing 0.12 mg of gangliosides (N-acetylsialic acid, 23.5 %; hexose (as galactose) 20 %) were added cerebrosides (galactose, 21.0 %) in different amounts. Bial's method was performed according to the standard procedure. The absorbancy of galactose was subtracted from the absorbancy reading (method I) or eliminated by reading at two wavelengths (method II).

Material	$(A_s)_{575 \text{ m}\mu}$	$(A_s)_{670 \text{ m}\mu}$	Amount of N-acetyl-sialic acid fond (μg)		Amount of N-acetyl-sialic acid calc. (μg)
			Method I	Method II	
Gangliosides, 0.12 mg	0.524	0.148	28.14	28.20	28.20
+ cerebrosides, 0.50 mg	0.581	0.204	28.00	28.28	28.20
+ cerebrosides, 1.00 mg	0.636	0.260	27.78	28.20	28.20
+ cerebrosides, 2.00 mg	0.693	0.326	23.98	27.53	28.20
+ cerebrosides, 4.00 mg	0.707	0.350	12.01	26.78	28.20

lengths. The amount of sialic acid can then be calculated from the following simple equation:

$$\frac{\text{sialic acid}}{(\text{mg}/5 \text{ ml})} = \frac{(A_s)_{575} - (A_s)_{680}}{(a_s)_{575} - (a_s)_{680}}.$$

As seen from Table 7, the agreement with the calculated amounts of sialic acid was good, when this procedure was adopted. It must here be emphasized that the described method has to be performed at a constant band width and that the ratio $(a_s)_{575}/(a_s)_{680} = 1$ for galactose cannot be used without checking.

If sialic acid is mixed with a sugar other than galactose the absorption curve of the latter must first be determined and afterwards the second wavelength chosen. Sometimes it is not possible to choose the second wavelength so that the ratio of a_s at the two wavelengths = 1 (e.g. for glucose and mannose). Then the equation already described has to be used (60). The method is also applicable when more than one sugar occurs together with sialic acid in the sample, if its nature and percentage are known. However, the method is only fit for use, when the ratio of the absorbancy indices of sialic acid and the other carbohydrates is independent of the concentration at the two wavelengths. All the carbohydrates tested satisfied this requirement except sorbose and fructose. Thus, Bial's method is not applicable, when sialic acids are mixed with ketohexoses in a sample. By substituting resorcinol for orcinol, however, it was possible to estimate such mixtures.

Sample blank

The method worked out was adopted for the determination of sialic acid in intact tissues. Often the tissues had a self-colour, which led to too high values of sialic acid. When the method was used for lipid extracts the colour of the unsaturated fatty acids was pronounced. It was thus necessary to compensate for the nonspecific colour by running a sample blank. As the sialic acids undergo decomposition at heating in mineral acids, which is inhibited by the orcinol, a sample blank has also been used for the standard samples of sialic acid. The sample blank was subtracted from the test sample before the calculation of the amount of sialic acid was made with the equation described.

Accuracy of the method

The standard deviation in the method has been calculated from thirty double determinations of 40 μg N-acetylsialic acid. The same calculation has also been done on thirty duplicates of lipid extracts from brain emulsified in potassium hydroxide. The determinations were performed over a period of four months. As the accuracy of the method was greatest when the absorbancy was about 0.700 (61), samples with an absorbancy of 0.500–0.900 were chosen.

	N-acetylsialic acid 40 μg	Lipid extracts from grey matter
Mean absorbancy	0.747	0.787
Standard deviation	± 0.0075	± 0.0187
Percentage error	± 1.0	± 2.4

The appreciably greater standard error of observation found for the lipid extracts is mainly due to difficulties in obtaining homogeneous solutions. As double determinations have been done, the standard error of the analysis is not larger than $\pm 1.7\%$.

Summary

A brief survey is given of the occurrence and the probable structure of sialic acids as well as the nomenclature used by different researchers.

The optimal conditions for the quantitative estimation of sialic acid with orcinol-hydrochloric acid (Bial's reagent) has been investigated. In the original reagent Fe^{3+} was used to increase the sensitivity of the reaction, but it was found that Cu^{2+} was at least as efficient as Fe^{3+} .

Three different sialic acids were tested: N-acetylsialic acid, N,O-diacetylsialic acid and N-glycolysialic acid. The molar absorptancy index of N-glycolysialic acid was about 20% higher than the indices of the other two.

The colour formation of sialic acids is influenced by other carbohydrates. The commonly occurring sugars in animal and plant materials were tested and the molar absorptancy indices determined. Under the conditions applied Beer's law is valid only within a small range for most of the carbohydrates. It is, however, possible to compensate the absorptancies of other carbohydrates by reading the samples at two wavelengths.

The nature of the colour formation is discussed. The available data show that Bial's reaction is both a furfural and a ketose reaction.

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Note added in proof.—Since the submission of this paper, Kuhn and Brossmer (Chem. Ber. 89 (1956), 2471) have succeeded in isolating N-acetyl-D-glucosamine by heating lactaminic acid with pyridine and nickel acetate. Further, lactaminic acid proved to be identical with N-acetylsialic acid prepared by Blix. Their new elementary analysis data of lactaminic acid also point in favour of the formula $\text{C}_{11}\text{H}_{19}\text{NO}_8$.

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